AD_____

Award Number: DAMD17-00-1-0188

TITLE: Novel Mechanisms by which Estrogen Induces Antiapoptosis in Breast Cancer

PRINCIPAL INVESTIGATOR: Ellis R. Levin, M.D.

CONTRACTING ORGANIZATION: University of California
Irvine, CA 92697-1875

REPORT DATE: August 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Final (10 Jul 2000 - 9 Jul 2004)

4. TITLE AND SUBTITLE
Novel Mechanisms by which Estrogen Induces Antiapoptosis in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0188

6. AUTHOR(S)

Ellis R. Levin, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California
Irvine, CA 92697-1875

E-Mail: ellis.levin@med.va.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We investigated the mechanisms of membrane estrogen receptor signaling to breast cancer. We discovered that serine 522 of mouse ERα is required for membrane localizaton. Importantly, when expressed in MCF-7 cells, this S522A mutant ERα heterodimerizes with endogenous ERα , sequestering it from the membrane and inhibiting ERK activation by E_2. This mutant has no effect on endogenous nuclear ER. We found that cyclin D_1 synthesis, Cdk4 activity and G_1/S progression is stimulated via ERK and inhibited by S522A mutant ERα expression in MCF-7 (Mol Cell Biol 23(3):1633-46, 2003). We also found that estradiol signaling to ERK involves transactivation of the EGF receptor, through $G_{q}\alpha$ and $G_{i}\alpha$ signaling to Src, Mmp activation and HB-EGF liberation. Only the E domain of ERα is required for these events initiated at the plasma membrane (J Biol Chem 278:2701-12, 2003) More recently, we showed that BRCA1 inhibits membrane ER and growth factor signaling through ERK to cell proliferation and this is lost when BRCA1 is mutated (MCB, July 2004, In press).

14. SUBJECT TERMS
Estrogen, anti-apoptosis, cell membrane estrogen receptor, signaling

15. NUMBER OF PAGES
63

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Estrogen promotes an increased incidence of breast cancer in women, while anti-estrogen therapy both limits recurrences and prevents the development of primary disease in genetically predisposed individuals. The purpose of the studies proposed in our grant is to determine the cellular mechanisms by which estrogen enhances the survival of breast cancer.

Body

In conjunction with the statement of work, we continue to determine mechanisms by which estradiol (E_2) and membrane estrogen receptors (ER) act as survival and growth stimulus for breast cancer. We found that serine 522 of mouse ERα is necessary for plasma membrane localization. When expressed in MCF-7 or 2R-75-1 cells, a mutant S522A ERα heterodimerizes and sequesters the membrane ER but not the nuclear ER, leading to ERK downregulation (but no effect on ER E-transcription). We showed that E_2-induced ERK importantly contributed to cyclin D_1 synthesis, increased Cdk4 activity against the retinoblastoma protein (inactivating phosphorylation), and subsequent G_1/S cell cycle passage. This was all markedly inhibited by expression of the S522A mutant ERα in the breast cancer cells. The data mechanistically support a role for ERK and membrane ER in mediating breast cancer proliferation and survival, and validate a novel reagent to specify membrane-initiated versus nuclear-initiated signaling by estrogen in breast cancer Mol Cell Biol 23:1633-46, 2003).

We also determined the mechanism by which membrane ER signals to ERK in breast cancer. This involves a cross talk to the EGF receptor, which is transactivated by the membrane and not nuclear ER. In fact, it is the E domain of ERα that is sufficient to accomplish this. E_2 ligation of membrane ERα causes a $G_q\alpha$ and $G_i\alpha$-dependent activation of Src via PKC and calcium signaling. This leads to Src-induced matrix metalloproteinase 2 and 9 cleavage/activation and liberation of Hb-EGF. Hb-EGF then binds and activates EFR, leading to both ERK and PI_3K upregulation in MCF-7 cells (J Biol Chem 278:2701-12, 2003). This was also reviewed in Mol Endo 17:309-17, 2003.

We also determined that signaling from the membrane receptor through PI_3 kinase resulted in the upregulation of 250 genes in the endothelial cell. This indicates that referable to the tumor vasculature, transcription of important target genes that participate in angiogenesis and other processes is modulated by the membrane ER signaling (J Biol Chem 277:50768-75, 2002).

Very recently, we addressed the issue of whether intact BRCA1 interacts with membrane ER function to prevent signaling to cell proliferation and survival. In initial studies, we found that in MCF-7 and ZR-75-1 cells, that expression of intact BRCA1 inhibits E2-induced ERK and cell proliferation over a 72-hour period. In contrast, three mutant BRCA1 proteins found in women with breast cancer do not affect these functions of ER. Intact BRCA1 also inhibited EGF-induced ERK and cell proliferation. Our studies indicate that BRCA1, in the setting of E2, induces a MAP kinase phosphatase, MKP-1 and enhances phosphatase activity to downregulate

ERK. Furthermore, expression of membrane localized or nuclear localized ERα supports E_2-induced proliferation in the absence of the other pool of ER (Mol Cell Biol, In press, July 2004).

Most recently, we have found that the endogenous ERα + ERβ at the plasma membrane of breast cancer cells exist predominantly as homodimers in the presence of E_2, and secondarily as ERα/ERβ heterodimers. We showed that dimmer formation is necessary for signaling to ERK, PI_3 kinase and cAMP, G protein activation and EGFR transactivation. ER dimer-induced signaling also is needed for the cell survival effect of E_2/ER in breast cancer (Mol Endocrinology, In press).

Key Research Accomplishments

- Identification of a structural determinant (serine 522) that is necessary for membrane localization of ERα.
- Development of a new specific reagent, a mutant ERα (S522A) that only downregulates endogenous membrane ER function.
- Proving that signaling through ERK by membrane ER is important for cell cycle progression of breast cancer.
- Elucidation of the important cross talk steps between membrane ER and the EGF receptor, resulting in breast cancer proliferation.
- Identification of a new tumor suppressor function for intact BRCA1, lost when mutated, thus predisposing to breast cancer development.

Reportable Outcomes

Abstracts and Presentations

1. Razandi M, Alton G, Pedram A, Shonshani S, Levin ER. Serine 522 of mouse estrogen receptor alpha is essential for membrane localization, signaling and cell biology. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.

2. Pedram A, Razandi M, Park ST, Levin ER. Proximal events in membrane estrogen receptor signaling requires G-protein induced transactivation of the EGF receptor. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.

3. Razandi M, Pedram A, Rosen E, Levin ER. Wild type BRCA1 inhibits membrane ER signaling to ERK and breast cancer cell proliferation. Presented at the 85th Annual Meeting of the Endocrine Society, Philadelphia, PA, June 2003.

4. Razandi M, Pedram A, Levin ER. ERα exists and functions as a dimer at the plasma membrane. Presented at the 86th Annual Meeting of the Endocrine Society, New Orleans, LA, June 2004.

Manuscripts

1. Pedram A, Razandi M, Aitkenhead M, Hughes CCW, Levin, ER. Integration of the non-genomic and genomic actions of estrogen: membrane initiated signaling by steroid (MISS) to transcription and cell biology. J Biol Chem 277(52):50768-50775, 2002.

2. Razandi M, Pedram, A, Parks S, Levin ER. Proximal events in ER signaling from the plasma membrane. J Biol Chem 278:2701-2712, 2003.

3. Levin ER. Bi-directional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 17:309-17, 2003.

4. Razandi M, Alton G, Pedram A, Ghonshani S, Webb D, Levin ER. Identification of a structural determinant for the membrane localization of ERα. Mol Cell Biol 23(3): 1633-1646, 2003.46, 2003.

5. Razandi M, Pedram A, Rosen E, Levin ER. BRCA1 inhibits membrane estrogen and growth factor receptor signaling to cell proliferation in breast cancer. Mol Cell Biol 24:(In press).

6. Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER. Plasma membrane estrogen receptors exist and functions as dimers plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol (In press).

Conclusions

Estradiol signaling from membrane ER is important to breast cancer cell survival and growth. The ERK pathway is very important in this regard, and activation of ERK by membrane ER requires cross talk to the EGF receptor. These results justify developing ER antagonists that selectively act at the cell membrane, to help prevent and treat breast cancer.

References
None.

Appendices
Five articles attached.

THE JOURNAL OF BIOLOGICAL CHEMISTRY APPENDIX 1 Vol. 277, No. 52, Issue of December 27, pp. 50768–50775, 2002
Printed in U.S.A.

Integration of the Non-genomic and Genomic Actions of Estrogen

MEMBRANE-INITIATED SIGNALING BY STEROID TO TRANSCRIPTION AND CELL BIOLOGY*

Received for publication, October 2, 2002
Published, JBC Papers in Press, October 7, 2002, DOI 10.1074/jbc.M210106200

Ali Pedram‡§, Mahnaz Razandi‡§, Mark Aitkenhead‡§¶, Christopher C. W. Hughes‡¶, and Ellis R. Levin‡§‖**

From the ‡Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822 and the Departments of §Medicine, ‖Pharmacology, and ¶Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717

Estrogen binds to receptors that translocate to the plasma membrane and to the nucleus. The rapid, non-genomic actions of this sex steroid are attributed to membrane action, while gene transcription occurs through nuclear receptor function. However, gene transcription can also result from estrogen signaling initiated at the membrane, but the relative importance of this mechanism is not known. In vascular endothelial cells (EC), estradiol (E_2) activates several kinase cascades, including phosphatidylinositol 3-phosphate (PI3K)/Akt, a signaling pathway that impacts EC biology. We determined here by DNA microarray that 40-min exposure to E_2 significantly increased 250 genes in EC, up-regulation that was substantially prevented by the PI3K inhibitor, LY294002. This coincided with maximum E_2-induced PI3K activity at 15–30 min. An important vascular gene strongly up-regulated by E_2 in our array produces cyclooxygenase-2 (Cox-2). In cultured EC, E_2 induced both *Cox-2* gene expression and new Cox-2 protein synthesis by 40 and 60 min, respectively, and rapidly stimulated the secretion of prostaglandins PGI_2 and PGE_2. The up-regulation of gene expression reflected transcriptional transactivation, shown using *Cox-2* promoter/luciferase reporters in the EC. Soluble inhibitors or dominant negative constructs for PI3K and Akt prevented all these actions of E_2. Functionally, EC migration was induced by the sex steroid, and this was significantly reversed by NS-398, a Cox-2 inhibitor. Gene transcription and cell biological effects of estrogen emanate from rapid and specific signaling, integrating cell surface and nuclear actions of this steroid.

Estradiol (E_2)[1] and other steroid hormones are traditionally considered to transactivate target genes after binding nuclear receptors (1). However, E_2 also has rapid, non-genomic effects (2–4), and these have recently been attributed to cell membrane-initiated signaling. At the cell surface, a small population of ER binds E_2 and activates G proteins (5–7). Multiple signaling pathways are then rapidly stimulated by E_2 in target cells that express endogenous ERα and ERβ, and these pathways have been linked to discrete cellular actions of the steroid (8–11). In this respect, a truncated MTA1 protein was recently found to be highly expressed in aggressive breast cancer (12). This protein sequesters ER away from the nucleus and strongly reduces E_2-activated transcription yet promotes increased ERK signaling and aggressive behavior of the tumor. It is proposed therefore that the integration of cell surface and nuclear signaling impacts overall cell biology (13).

Signaling from the membrane leads to the post-translational modification of important structural and functional proteins in the cell. In EC, E_2 activates the p38-MAPKAP-2 kinase pathway; MAPKAP-2 phosphorylates and modifies the function of heat shock protein 27 (14). This important modification leads to the morphological preservation and survival of the EC and stimulates primitive capillary tube formation. In breast cancer, E_2 acts as a cell survival factor, in part by preventing chemotherapy or radiation-induced JNK activation (9). JNK phosphorylates and inactivates Bcl2 and Bcl-xl, leading to the assembly of the apoptosome and caspase-mediated cell death. By preventing JNK activation and Bcl2/Bcl-xl phosphorylation, E_2 rescues the breast cancer cells (9). This provides a mechanism for the ability of E_2 to oppose therapeutic interventions in this malignancy.

In addition to post-translational protein effects, E_2 is recognized to stimulate transcription through signaling typically initiated at the membrane. As precedent for this effect of E_2, growth factor tyrosine kinase receptors (insulin-like growth factor-1 receptor and epidermal growth factor receptor) exist in the plasma membrane and signal through common kinase cascades to gene transcription. E_2 effects may involve the G protein-initiated, signaling-induced synthesis or activation of transcription factors. E_2 stimulates c-*fos* through ERK- or PI3K-dependent pathways (15–17), the *BCL-2* gene through the modification of Sp-1 transcription factor (18), and the *prolactin* gene through ERK (19). E_2 activation of PI3K in EC results from the membrane ER-p85 regulatory subunit (PI3K) association, and this signaling to nitric oxide production rescues rats from ischemia-reperfusion injury (20). However, insight is largely lacking as to the full extent and importance of the specific integration of membrane signaling and nuclear effects of the sex steroid.

We therefore identified the transcriptional targets in EC that are rapidly up-regulated from E_2 signaling through the activation of PI3K. We also describe in depth the ability of

* This work was supported by grants from the Research Service of the Department of Veterans Affairs, Avon Products Breast Cancer Research Foundation, Department of Defense Breast Cancer Research Program (Grant BC990915) and the National Institutes of Health Grant HL-59890 (to E. R. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Medical Service (111-I), Long Beach Veterans Administration Medical Center/UC-Irvine, 5901 E. 7th St., Long Beach, CA 90822. Tel.: 562-826-5748; Fax: 562-826-5515; E-mail: ellis.levin@med.va.gov.

[1] The abbreviations used are: E_2, estradiol; Cox, cyclooxygenase; EC, endothelial cells; ER, estrogen receptor; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPKAP, mitogen-activated protein kinase activated protein kinase; PI3K, phosphatidylinositol 3-phosphate; RT, reverse transcriptase; PGI_2, prostaglandin I_2; PGE_2, prostaglandin E_2; NO, nitric oxide; EGR, early growth response; bFGF, basic fibroblast growth factor; DN, dominant negative; MISS, membrane-initiated steroid signaling; PTTG, pituitary tumor transforming gene; BAEC, bovine aortic endothelial cell.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

APPENDIX 2

Vol. 278, No. 4, Issue of January 24, pp. 2701–2712, 2003
Printed in U.S.A.

Proximal Events in Signaling by Plasma Membrane Estrogen Receptors*

Received for publication, June 8, 2002, and in revised form, October 11, 2002
Published, JBC Papers in Press, November 5, 2002, DOI 10.1074/jbc.M205692200

Mahnaz Razandi‡, Ali Pedram‡, Steven T. Park‡, and Ellis R. Levin‡§¶

From the Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822 and the Departments of ‡Medicine and §Pharmacology, University of California, Irvine, California 92717

Estradiol (E2) rapidly stimulates signal transduction from plasma membrane estrogen receptors (ER) that are G protein-coupled. This is reported to occur through the transactivation of the epidermal growth factor receptor (EGFR) or insulin-like growth factor-1 receptor, similar to other G protein-coupled receptors. Here, we define the signaling events that result in EGFR and ERK activation. E2-stimulated ERK required ER in breast cancer and endothelial cells and was substantially prevented by expression of a dominant negative EGFR or by tyrphostin AG1478, a specific inhibitor for EGFR tyrosine kinase activity. Transactivation/phosphorylation of EGFR by E2 was dependent on the rapid liberation of heparin-binding EGF (HB-EGF) from cultured MCF-7 cells and was blocked by antibodies to this ligand for EGFR. Expression of dominant negative mini-genes for $G\alpha_q$ and $G\alpha_i$ blocked E2-induced, EGFR-dependent ERK activation, and $G\beta\gamma$ also contributed. G protein activation led to activation of matrix metalloproteinases (MMP)-2 and -9. This resulted from Src-induced MMP activation, implicated using PP2 (Src family kinase inhibitor) or the expression of a dominant negative Src protein. Antisense oligonucleotides to MMP-2 and MMP-9 or ICI 182780 (ER antagonist) each prevented E2-induced HB-EGF liberation and ERK activation. E2 also induced AKT up-regulation in MCF-7 cells and p38β MAP kinase activity in endothelial cells, blocked by an MMP inhibitor, GM6001, and tyrphostin AG1478. Targeting of only the E domain of ERα to the plasma membrane resulted in MMP activation and EGFR transactivation. Thus, specific G proteins mediate the ability of E2 to activate MMP-2 and MMP-9 via Src. This leads to HB-EGF transactivation of EGFR and signaling to multiple kinase cascades in several target cells for E2. The E domain is sufficient to enact these events, defining additional details of the important cross-talk between membrane ER and EGFR in breast cancer.

Steroid hormones such as estrogen are essential to the development and reproductive functions of prokaryotic and eukaryotic organisms. Traditionally, steroid hormone action was exclusively attributed to the binding of nuclear receptors and the subsequent transactivation of target genes that led to cell biological effects (1). More recently, it has become clear that steroids rapidly act on cells, in seconds to minutes, effects that are classified as "nongenomic" (reviewed in Ref. 2). For estrogen, this has been attributed in most cells to binding a population of receptors that exists within caveolar rafts and other domains in the plasma membrane (3–5). It is at the plasma membrane that estradiol (E2)[1]-liganded estrogen receptors (ER) physically associate with the scaffold protein, caveolin-1 (5), and a variety of proximal signaling molecules, including G proteins (6, 7), Src and Ras (8, 9), and B-Raf (10). This results in the activation of cascades of signal transduction, mainly evolving from G protein activation. Comparable with many other G protein coupled receptors (GPCR), G protein activation by ER (6, 7) leads to the stimulation of phospholipase C (11), protein kinase C (12), ERK (9), and phosphatidylinositol 3-kinase and nitric-oxide synthase (13). These positive signaling effects are cell context-specific, and in some cells, estrogen inhibits cytokine-related signal transduction to cell differentiation, proliferation, migration, or cell death (14–17).

What is the nature of the membrane ER, and how does it enact signal transduction? Current evidence favors the idea that the membrane and nuclear ER are the same protein. Antibodies directed against many epitopes of the classical ERα receptor identify membrane ER by immunocytochemistry (18). Expression of antisense DNA to the "nuclear" ER also abrogates the detectable expression of membrane ER in cells containing endogenous receptor (19). In CHO cells, expression of a single cDNA for either ERα or ERβ produces both membrane and nuclear receptor populations and results in E2 activation of signal transduction from the membrane (6). In many cell types, endogenous membrane ER have been identified (15, 18, 20) and appear to reflect the localization of receptors that also have the capacity to translocate to the nucleus. The structural aspects of the membrane ER that allow it to activate signaling molecules are not well defined. Assuming that the sequence of the nuclear receptor is the same as the membrane ER, there is no catalytic or kinase sequence inherent to the structure. Recent evidence favors the idea that the E domain of the membrane ER is essential (and perhaps sufficient) for activation of the ERK cascade (5), leading to cell survival (17). Additionally, the AF-1 domain of ERα has been identified to interact with the adapter protein, Shc, in whole cell homogenates (21). Thus, the

* This work was supported by a grant from the Research Service of the Department of Veteran's Affairs, a grant from the Avon Products Breast Cancer Research Foundation, Department of Defense Breast Cancer Research Program Grant BC990915, and National Institutes of Health Grant HL-59890 (to E. R. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Medical Service (111-I), Long Beach VA Medical Center/UC-Irvine, 5901 E. 7th St., Long Beach, CA 90822. Tel.: 562-826-5748; Fax: 562-826-5515; E-mail: ellis.levin@med.va.gov.

[1] The abbreviations used are: E2, estradiol; ER, estrogen receptor(s); EGF, epidermal growth factor; EGFR, EGF receptor(s); GPCR, G protein-coupled receptor(s); MMP, matrix metalloproteinase(s); PLC, phospholipase C; PKC, protein kinase C; HB-EGF, heparin-binding epidermal growth factor; TGF, transforming growth factor; EC, endothelial cell(s); CHO, Chinese hamster ovary; ASO, antisense oligonucleotide(s); MSO, scrambled antisense oligonucleotide(s).

E_2-induced PI3K activation to up-regulate one specific gene, *Cox-2*, leading to the production of the enzyme, secretion of products of Cox-2 activation, and EC migration. This approach can be used to identify programs of gene activation that result from membrane-initiated steroid signaling (MISS)[2] by E_2.

MATERIALS AND METHODS

PI3K Activity Assay—EC were incubated with/without 10 nM E_2 and 10 μM LY294002 for up to 6 h. Cells were then lysed, and the lysates pelleted then dissolved in SDS sample buffer, boiled, separated, and transferred onto nitrocellulose. Phosphorylated Akt was detected using phospho-specific monoclonal antibodies (Santa Cruz Biotechnology) and the ECL Western blot kit. Equal samples from the cells were also immunoprecipitated, and immunoblots of the precipitated kinase protein from each experimental condition were determined to show equal gel loading. All experiments were repeated two or three times.

DNA Arrays—Human umbilical vein endothelial cells were incubated without or with 10 nM E_2 with or without the specific PI3K inhibitor, LY294002 (10 μM), or LY294002 alone, for 40 min. For validation, the experiment was repeated a second time. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and by RNeasy columns (Qiagen, Valencia, CA). Total RNA was adjusted to 1 μg/μl, and first-strand cDNA, followed by double-stranded cDNA was synthesized from poly(A)$^+$ mRNA by the Microarray Facility at the University of California, Irvine. This was done using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and poly(T) nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction (IVT) using the BioArray High-Yield RNA transcript labeling kit (T7) (Enzo Diagnostics, Farmingdale, NY). 15 μg of the biotin-tagged cRNA was fragmented to strands of 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). 10 μg of the fragmented cRNA was hybridized with rotation at 45 °C for 16 h to probe sets present on an Affymetrix human U95a array (Affymetrix, Santa Clara, CA). The arrays were automatically washed and stained with streptavidin-phycoerythrin. Probe arrays were then scanned on a Hewlett-Packard GeneArray scanner. Affymetrix Microarray Suite 5.0 was used to quantify and analyze the average difference in intensities between conditions of the experiment, for each represented gene. The comparisons between conditions were outputted as -fold increase or decrease, or no change, and data were compared with determine which genes were E_2-responsive in a PI3K-dependent fashion. This output was then inserted into a stringent, Bayesian-based statistical analysis program (Cyber-T) (21), available as a web interface at the University of California at Irvine. The upper 2.5% of genes identified as being significantly different by Cyber-T (and in agreement with the Affymetrix analysis) are presented in table form.

RT-PCR and Reporter Assays—Validation of differential expression was performed by RT-PCR for four genes identified in the microarray and was standardized to glyceraldehyde-3-phosphate dehydrogenase. cDNA was prepared from 3 μg of total RNA isolated from EC, primed with random hexamers (Invitrogen), and reverse-transcribed with Superscript II (Invitrogen) per the manufacturer's instructions. The primers were prepared by Invitrogen for the following sequences; *Cox-2*, TGGGAAGCCTTCTCTAACCTCTCCT and CTTTGACTGTGGGAGGATACATCTC; *CREM*, TGGAAACAGTTGAATCACAG; and CTACTAATCTGTTTTGGGAG; *EGR2*, CAGTACCCTGGTGCCAGCTG and TGTGGATCTCTCTGGCACGG; *JUN-B*; CCGGATGTGCACGAAAATGGAACAG and ACCGTCCGCAAAGCCCTCCTG; glyceraldehyde-3-phosphate dehydrogenase; ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA. PCR reactions were performed using 200 nM of primers with 2 μl of cDNA in 50 μl of Platinum PCR supermix (Invitrogen). After an initial denaturation step of 94 °C for 4 min, 25–35 cycles of 94 °C for 30 s, 55–61 °C for 45 s, 72 °C for 30–45 s, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The cycle number and annealing temperature was adjusted for each of the genes amplified.

For reporter assays, BAEC were transiently transfected with 10 μg of the PGL-3 plasmid containing 1.8 kb of the human *Cox-2* promoter driving a luciferase reporter fusion protein (22), kindly provided by Dr. David Dixon (Vanderbilt). The liposome-mediated transfection was carried out as previously described (6, 9), and the cells were recovered in serum overnight, then synchronized without serum over 24 h before experiments (in phenol red-free medium). Cells were then incubated for 6 h with10 nM E_2 with or without either LY294002 (10 μM), a soluble inhibitor of NFκB (SN50M), or the corresponding inactive control (SN50), each at 20 μM. All inhibitors were from Calbiochem (San Diego, CA). Assays were quantified by luminometer, and the results were adjusted for expression of co-transfected *Renilla* luciferase. Triplicate determinations per condition were carried out in each of two experiments. The combined data were analyzed by analysis of variance plus Schefe's test, at a $p < 0.05$ level of significance.

Cox-2 Protein and PGI_2 Secretion—For protein synthesis, BAECs were exposed to 0.1–10 nM E_2 for 60 min, preceded where indicated for 30 min by incubation with ICI 182,780 (1 μM), LY294002 (10 μM), or wortmannin (PI3K inhibitor) (100 nM). Additional BAECs were either transiently transfected with dominant negative myc-tagged pMT2-AH-Akt (kindly provided by Dr. Julian Downward) (23), or dominant negative PI-3K p85 subunit (pcDNA3-delta p85, lacking residues 478–513) (kindly provided by Dr. Barry Posner) (24), as previously described (25), or with pcDNA3 as control. The cells were recovered and synchronized over 24 h then incubated with E_2.

For PGI_2 (measured as 6-keto-PGF1α) and PGE_2 secretion studies, BAEC were incubated for 0–240 min with E_2, to determine time of peak secretion. This was found to be 30 min, as subsequent time points reflected both secretion and accumulation. Additional studies were then conducted at 30 min. BAEC were incubated with/without 10 nM E_2, with or without inhibitors, including NS-398 (5 μM) (Cox-2 inhibitor), or SC-560 (20 nM) (Cox-1 inhibitor). Each condition was run in duplicate. The incubation media were collected and concentrated by lyophilization, and prostaglandin concentrations were determined by enzyme-

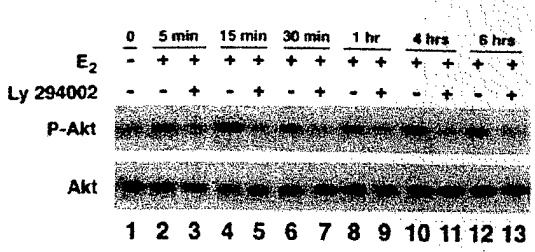


FIG. 1. **Time course of PI3K activation in endothelial cells by estradiol (E_2).** Cells were incubated with 10 nM E_2 with or without 10 μM LY294002 for the times indicated, and Akt phosphorylation as a function of PI3K activity was determined by Western blot. Immunoblot of total Akt protein is shown *below* each condition. A representative study of two completed is shown.

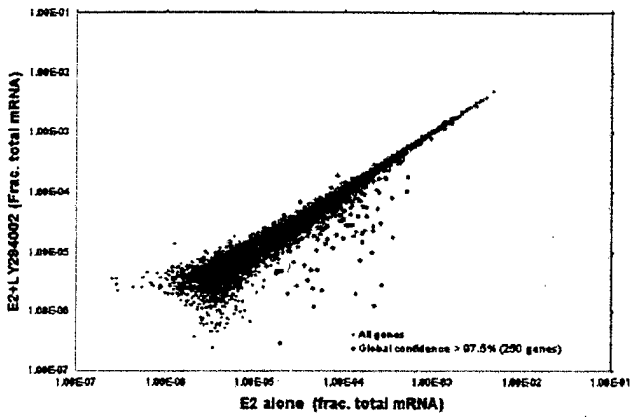


FIG. 2. **Estrogen-induced genes in endothelial cells, dependent upon phosphatidylinositol 3-kinase signaling.** EC were incubated/not incubated with 10 nM E_2 with or without LY294002 (PI3K inhibitor), or LY294002 alone, for 40 min. Total RNA from the four conditions was then extracted for purposes of microarray analysis after cDNA/cRNA synthesis (see "Materials and Methods"). The figure is the output from CyberT analysis, a stringent, Bayesian-based statistical analysis program. Duplicate determinations for all conditions were utilized for the analysis. Genes in *red* are the upper 2.5% of all genes positively regulated by E_2 and mostly inhibited by LY294002.

[2] Terminology to replace "non-genomic" effects of steroids, as suggested by the Consensus Working Group at the FASEB conference on Membrane Steroid Receptors, Aspen, CO, June 22–27, 2002.

TABLE I

Genes significantly up-regulated by estradiol in a PI3K-dependent fashion

Genes that were induced at least 2-fold in endothelial cells after 40-min exposure to E_2 but were not significantly induced after exposure to E_2 and Ly294002 are listed. Differentially expressed genes were identified by both Affymetrix analysis software and the Cyber T (Bayesian) statistical analysis program.

Description		Accession #	Fold Increase
Transcription Factors			
early growth response-3 protein	EGR-3	X63741	70.49
early growth response 2 protein	EGR-2	J04076	66.84
Zinc finger protein 36	GOS24	M92843	20.25
Areb6 Transcription factor	AREB6	D15050	18.47
early growth response 1 protein	EGR 1	S81439	10.10
DEC-1 transcription factor	BHLHB2	AB004066	8.64
zinc finger protein 137	ZFP137	U09414	7.02
Max dimerization protein	MAD	L06895	7.01
myogenic factor 5	Myf-5	X14894	6.35
jun B proto-oncogene	JunB	X51345	6.24
activating transcription factor 3	ATF3	L19871	6.01
CREM-beta;	CREM	S68134	5.73
c-fos oncogene	c-fos	V01512	5.05
zinc finger protein	FDZF2	U95044	4.67
msg-related gene 1	mrg1	U65093	4.55
immediate early protein	ETR11	M62831	4.34
Forkhead box C1	FKHL7	AF078096	4.18
B Cell lymphoma protein 6	bcl-6	U00115	4.16
c-jun oncogene	c-JUN	J04111	3.99
c-myc oncogene	c-myc	V00568	3.86
DNA damage inducible transcript 3	DDIT3	S62138	3.76
immediate-enrly gene X1	IEX-1	S81914	2.93
special AT-rich sequence binding protein 1	SATB1	M97287	2.91
butyrate response factor 2 (EGF-response factor 2)	ERF-2	X78992	2.77
Inhibitor of growth like-1	ING1L	AB012853	2.68
KRAB zinc finger	ZNF75	S67970	2.66
Regulator of G protein signaling 2	RGS2	L13463	2.44
Hairy enhancer of split1	HES1	L19314	2.40
basic helix-loop-helix transcription factor 15	TCF15	U08336	2.35
basic helix-loop-helix transcription factor 2	TCF2	U19345	2.30
Sprouty 1	spry-1	AF041037	2.30
positive elongation transcription factor b	P-FEBb	AF048732	2.29
Macrophage stimulating pseudogene 9	MSTP9	U28055	2.23
IkappaB alpha	IKBA	M69043	2.23
Zinc finger protein 17	ZNF17	AF041259	2.22
Multi drug resistance	MDR1	X58723	2.18
hox 5.1 protein	HSX5.1	X17360	2.11
alpha-Pal	NRF1	U02683	2.10
Sp4 transcription factor	SPR-1	X68561	2.01
myocyte-specific enhancer factor 2A	MEF2A	U49020	2.00
Mesenchyme Fork Head-1	MFH-1	Y08223	2.00

Description		Accession #	Fold Increase
Signaling			
Cot/Tpl-2 kinase	MAP3K	D14497	13.64
GTP-ase	gem	U10550	11.34
protein-tyrosine phosphatase	CL 100	X68277	11.07
corticotrophin releasing factor receptor	CRHR1	X72304	7.97
putative topoisomerase-III	TOPO3	D87012	6.46
insulin receptor substrate-1	IRS-1	S62539	6.22
STAT-induced STAT inhibitor-2	STATI2	AF037989	5.55
protein tyrosine phosphatase	PTP-U2	Z48541	4.94
Sprouty 2	SPRY2	AF039843	4.58
Orphan G protein-coupled receptor	RDC1	U67784	4.55
SRC-like tyrosine kinase	FRK	U00803	4.46
glycerol kinase	GKTB	X78711	4.10
Toll like receptor 2	TIL4	AF051152	3.83
serine/threonine protein kinase	sgk	Y10032	3.71
G protein-coupled receptor	CNR1	U73304	3.59
Wnt-5a	Wnt-5a	L20861	3.51
PI3K regulatory 1	GRB1	M61906	3.01
GS3955	GS3955	D87119	2.97
Wnt-7a	WNT7a	D83175	2.87
chapsyn-110	DLG2	U32376	2.83
neurotrophic tyrosine kinase receptor 3	trkC	S76475	2.81
serum-inducible kinase	SNK	AF059617	2.68
P2Y6 receptor	P2Y6R	AF007893	2.67
intra-acrosomal protein	SP-10	S65583	2.63
SA (rat hypertension-associated) homolog	SA	X80062	2.61
type II interleukin-1 receptor	IL-1R2	X59770	2.58
Dual Specificity protein phosphatase 6	DUSP6	AB013382	2.54
guanine nucleotide exchange factor	SOS1	L13857	2.52
Nuclear Factor of Activated T cells x4	NFATx4	U85430	2.38
plasma membrane calcium ATPase	PMCA2	X63575	2.38
SNF1-like protein kinase	SNFLPK	U57452	2.36
MAP kinase phosphatase	MKP-2	U48807	2.33
inositol polyphosphate 4-phosphatase type II-alpha	IPP4P2A	U96922	2.31
multiple PDZ domain protein	MUPP1	AF093419	2.31
Chloride channel protein	CLCN3	X78520	2.19
putative g protein-coupled receptor	TM7SF1	AF027826	2.18
G protein-linked receptor	GPCR	L42324	2.16
kappa opioid receptor	OPRK1	L37362	2.12
thrombomodulin precursor	THBD	J02973	2.02
receptor phosphatase PCP-2	PCP-2	X97198	2.02

linked immunosorbent assay (Cayman Chemical). The study was repeated three times, with the results reflected in the *bar graph*.

EC Migration Studies—EC were grown to monolayer confluence on six-well plates and synchronized for 24 h in the absence of serum. A "wound" was created by scraping the monolayer with a single-edge razor blade, and cells were removed to the left of the wound. Serum-free Dulbecco's modified Eagle's medium containing 10 nM E_2 with or without NS-398, SC-560, LY294002, wortmannin, or alone as control was added to separate dishes of wounded EC for 24 h at 37 °C. The cells were then fixed in 3.7% formaldehyde and assessed for migration (14). BAEC migration was measured using an image analyzer system composed of an inverted microscope and a 20- to 24-inch digitizing board (Jandel Scientific, Corte Madera, CA) attached to a computer. The Sigma Scan program (Jandel) was used for analysis of measurements of the distance traveled by the cells within the calibrated area adjacent to the wound. Five measurements in each well were taken, and results from three separate experiments contributed to create the bar graph.

RESULTS AND DISCUSSION

Estrogen Stimulates PI3K Activity in EC—E_2 induced substantial Akt phosphorylation by 5 min, reflecting PI3K activation, because the phosphorylation was totally prevented by a PI3K inhibitor (Fig. 1). The peak activity occurred at 15 min and lasted for the 6-h duration of the experiment. We therefore carried out our array studies to assess genes rapidly induced by E_2 via PI3K signaling, based upon these results.

E_2 Rapidly Induces Many Genes via PI3K Activation—EC were incubated with 10 nM E_2 or without steroid (control) for 40 min, in the presence or absence of LY294002, a PI3K inhibitor. cRNA from each experimental condition was used for microarray gene analysis, as delineated under "Materials and Methods." The DNA array hybridization pattern was analyzed by both Affymetrix statistical software and the CyberT Bayesian-based program, and 250 genes were identified as being significantly up-regulated by E_2 (upper 2.5% of all genes) (Fig. 2). This occurred in both E_2-inducible and PI3K-reversible fashion. In comparing control cRNA (no treatment) to cRNA from cells treated with LY294002 alone, no differences were detected. We list the genes that were 1) up-regulated in this fashion by more than 2-fold and 2) identified to have some known function (Table I). In contrast, few genes were down-regulated by E_2 in the quiescent EC, and none depended upon PI3K/Akt activation, whereas several genes were stimulated by E_2 and further enhanced upon PI3K inhibition. Genes discussed in the text are given in boldface in Table I.

As might be predicted from the time chosen, many transcription factors were rapidly up-regulated. These included the *fos*, *myc*, and *jun* genes previously known to be stimulated by estrogen (15, 26, 27), which thereby validate our results. Here we extend the findings and implicate PI3K action in proto-oncogene up-regulation by E_2. This sex steroid was recently shown to activate c-*fos* transcription in MCF-7 cells, via a PI3K/Akt pathway, targeting the serum response factor motif in the proximal *fos* promoter (16). We also found stimulation of many transcription factors not previously known to be regu-

TABLE I—*continued*

Description		Accession #	Fold Increase
Biosynthesis/Enzymes			
cyclooxygenase-2	hCox-2	U04636	175.93
zinc finger protein 267	HZF2	X78925	3.91
alpha-2,8-polysialyltransferase	PST	L41680	3.70
preceruloplasmin (EC 1.16.3.1)	CP	M13699	3.69
sulfotransferase family 1C, member 1	SULT1C1	AB008164	3.20
Osteoblast specific cysteine rich protein	OSCP	AB008375	2.33
ADP-ribosyltransferase 3	ART3	U47054	2.29
glycerol-3-phosphate dehydrogenase 2	G3PD2	U36310	2.28
Retinol dehydrogenase	RODH	U89281	2.23
Phosphodiesterase 4B	PDE4B	L20971	2.21
Gamma-glutamylcysteine synthetase	GGCS	L35546	2.20
Hep27	Hep27	U31875	2.19
pseprocarboxypeptidase A2	proCPA2	U19977	2.18
Placental Protein 5	PP5	D29992	2.13
Cytokines and Chemokines			
GRO2 oncogene	MIP2A	M36820	38.94
interferon-beta-2	IFN-B2	X04430	11.98
insulin-like growth factor II	IGF2	J03242	9.20
TGFb inducible early protein	TEIG	AF050110	8.67
bone morphogenetic protein 2A	BMP2A	M22489	7.63
Thrombopoietin	TPO	L33410	4.97
CYR61 protein	CYR61	Y11307	4.91
pituitary tumor transforming gene protein 2	PTTG2	AF095288	4.81
inhibin beta-B-subunit precursor	INHBB	M31682	3.81
interferon	IFN	V00542	3.61
growth differentiation factor 5	CDMP1	X80915	3.53
heparin-binding epidermal gf-like growth factor	HEGFL	M60278	2.92
angiotensin II type 2 receptor	AT2	U20860	2.31
erythropoietin	EPO	X02158	2.22
leptin	OB	D63710	2.21
tachykinin 2 precursor, isoform beta	TAC2	U37529	2.18
interferon-related developmental regulator 2	IFNRP	U09585	2.16
connective tissue activating peptide III	PPBP	M54995	2.16
keratinocyte growth factor	FGF7	M60828	2.15
RANTES	RANTES	M21121	2.11
Ephrin B2	EFNB2	U81262	2.07
prostaglandin E2 receptor	PGER2	U19487	2.04
endothelial differentiation protein (edg-1)	EDG1	M31210	2.03
Orphan Receptors			
TR3 orphan receptor	NP1	L13740	97.28
mitogen induced nuclear orphan receptor	MINOR	U12767	25.60
neuron derived orphan receptor	NOR-1	D78579	4.07
Cell fate			
CAGR1	CAGR1	U38810	2.64
Bcl-2 like 11	BCL2L11	AF032457	2.52
Chromosome associated protein E	hCAP-E	AF092563	2.50
pleckstrin homology-like domain A1	PHLDA1	Z50194	2.39
Cullin gene family member	Hs-cul-4A	U58090	2.31
regulatory partner for cdk5 kinase	CDK5P35	X80343	2.27
N-myc downstream regulated	NDR1	D87953	2.11
Cell Structure/adhesion			
Chondroitin sulfate Versican	CSPG2	X15998	5.26
M-phase phosphoprotein 11	MPP11	X98260	3.44
crystallin	CRYA1	X14789	3.35
alpha-catenin	CTNNA2	M94151	3.23
vitronectin alpha subunit precursor	VNRA	M14648	2.78
Down syndrome cell adhesion molecule	CHD2-42	AF023450	2.67
Bicaudal 1	BICD1	U90030	2.27
cubilin	CUBN	AF034611	2.21
Immune response			
immunoglobulin heavy chain V(H)5	IHC-V(H)5	X58401	7.10
Human V beta Tcell receptor	TCRVB6S1	U03115	6.94
Semaphorin E	SEME	AB000220	6.94
CD28 ligand	B7-2	U04343	3.86
NK-associated transcript 3	NKAT3	X93595	3.59
VJ region immunoglobulin	IGL	AF043586	3.47
CD69 antigen	CD69	Z22576	3.10
phospholipase A2 activating protein	PLAP	AJ238243	2.94
HLA-DRB1	HLA-DRB1	M32578	2.81
Src-like adapter protein	SLAP	D89077	2.76
pre-T/NK cell associated protein (3Cl)	3Cl	L17328	2.71
PMA induced gene 1	APR	D90070	2.59
CD30 ligand	CD30L	L09753	2.59
cerebellar degeneration-associated protein	CDR1	M16965	2.51
complement factor H-related protein 4	FHR-4	X98337	2.17
MAGE-B2	MAGE-B2	U93163	2.08
Protein Tyrosine phosphatase, Non recpt2	PTPN2	M25393	2.07
C-reactive protein	PTX1	X56692	2.03
sialophorin CD43	CD43	X52075	2.01
Neuro			
proneurotensin/proneuromedin N	NTS	U91618	3.79
synaptobrevin 1 (SYB1)	SYB1	M36200	2.83
Uncategorized			
monocyte secretory protein	JE	M28225	8.19
tcar protein/PRPb homolog	BPLP	S83198	5.26
lethal (3) malignant brain tumor protein	l(3)mbt	U89358	3.67

lated by E_2; these genes are therefore implicated in further E_2 transcriptional action. In some situations, linked gene programs could be tentatively identified, based upon the existing literature. As an example, bone morphogenetic protein 2 (Table I, cytokines) stimulates osteoblast precursor-cell differentiation in part via up-regulating the AREB6 transcription factor (29). Estrogen induces osteoblast differentiation (30), and we found that E_2 stimulates both genes here, via a PI3K-induced mechanism. Another transcriptional target for AREB6 is the Na^+/K^+-ATPase gene (31). E_2 is known to stimulate the activity of this enzyme (32), potentially linking these observations to upstream signaling. As shown here, the HZF2 transcription factor is induced by E_2 in PI3K-dependent fashion (Table I). HZF2 has been reported to be up-regulated by nitric oxide (NO) (33), and E_2 strongly and rapidly stimulates NO production in EC *in PI3K-dependent fashion* (20). NO induction by E_2 prevents the deleterious blood vessel response to ischemia-reperfusion injury (20). Together, these results potentially identify a linked cell biological program in EC. Several members of the EGR family of transcription factors were identified as induced by E_2 in our array. Egr-1 is up-regulated (and important) in the response to acute and chronic vascular injury, where it may serve a protective function (34, 35). E_2 mitigates the acute injury response to carotid angioplasty (36), perhaps in part through inducing Egr-1 in the EC, as shown here.

Signaling molecules were also rapidly induced by E_2, including both kinases and phosphatases. Steroid and glucocorticoid-inducible kinase activity is known to be a target for PI3K/Akt signaling (37) and has roles in both steroid-induced memory (38) and sodium transport (39), which are both functions of E_2 (40, 41). We also found that, via PI3K, E_2 up-regulates *Cot/(Tpl-2)*, a transformation-associated factor and serine/threonine kinase. Akt phosphorylation of Cot induces NFκB-dependent transduction (42), important for various functions, including cell survival. E_2 potently inhibits hypoxia-induced EC apoptosis (14).

Genes coding for structural proteins, cytokines, or enzymes were identified as being stimulated by E_2. Two members of the NGFI-B subfamily of nuclear orphan receptors, *TR3* and *MINOR*, were rapidly and strongly induced by E_2 via PI3K. A cytokine gene, *CYR61*, was previously demonstrated to be up-regulated by E_2 in breast cancer but through an unknown mechanism (43). In EC, we implicate signaling via PI3K. Up-regulation of the *PTTG* gene by estrogen (found here) was previously shown to contribute to the pathogenesis of E_2-induced pituitary tumor formation and the stimulation of bFGF secretion (44). E_2 and bFGF are recognized angiogenesis factors (14, 45), and therefore a potential linkage in EC of PTTG-induced bFGF could be important for the recognized neovascularization function of E_2. The *PLAP* (phospholipase A2-activating protein) gene was found to be stimulated in our array (Table I). This protein has been reported to contribute to up-regulate the *Cox-2* gene and stimulate prostaglandin E_2 (PGE_2) production (46). Based on our results, we propose a

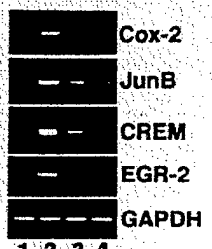


FIG. 3. **Validation of the microarray data by RT-PCR.** EC were incubated with 10 nM E_2 (*lane 2*), with 10 nM E_2 and LY294002 (PI3K inhibitor) (*lane 3*), with LY294002 alone (*lane 4*), or no treatment (*lane 1*) for 40 min. Total RNA from the four conditions was then extracted and reverse-transcribed to cDNA. PCR was performed using gene-specific primers (see "Materials and Methods") for *Cox-2*, *JunB*, *CREM*, and *EGR-2*, with glyceraldehyde-3-phosphate dehydrogenase as a control.

linkage of E_2-induced, PI3K-dependent up-regulation of *PLAP*, contributing to E_2-induced Cox-2 up-regulation and PG secretion/production (see below). Thus, many relevant genes are rapidly induced by this sex steroid in response to one signal pathway typically initiated at the plasma membrane.

Confirmation by RT-PCR of E_2-induced Gene Up-regulation—To confirm the array studies, we carried out semi-quantitative RT-PCR for several of the genes identified (Fig. 3). For the four genes examined (*Cox-2*, *JunB*, *CREM*, and *EGR2*), there was increased expression in EC after 40-min exposure to 10 nM E_2 (*lane 2*), compared with the control (no E_2) (*lane 1*). Furthermore, this increase was significantly abrogated by addition of the PI3K inhibitor, LY294002 (*lane 3*), whereas the inhibitor alone had no effect compared with control levels (*lane 4*). The results provide validation of the array data for these specific genes.

Cox-2 Gene and E_2/ER Interactions Result from Rapid Signaling by the Steroid—One of the genes strongly up-regulated by E_2 in our EC gene array codes for the Cox-2 enzyme (Table I). Cox-2 activity gives rise to pGI_2 and PGE_2 production, important for various aspects of vascular function (47, 48). To explore the interactions between E_2 signaling through PI3K to Cox-2 in greater depth, we first confirmed the array results by RT-PCR (Fig. 3). We then further investigated transcriptional regulation by E_2. We therefore expressed in BAEC a plasmid containing a 1.8-kb human *Cox-2* promoter driving a luciferase reporter. This was significantly responsive to 10 nM E_2, in a PI3K-dependent fashion (Fig. 4).

One known target for PI3K/Akt signaling is the activation of the NFκB transcription factor (49, 50). We found that a soluble NFκB inhibitor (but not its inactive control) completely reversed the E_2 stimulation of the *Cox-2* promoter (Fig. 4). These data support the microarray studies and the idea that the up-regulation of the *Cox-2* gene by E_2 is transcriptional. Furthermore, this E_2 action depends on signaling via PI3K and NFκB. NFκB binding sites are present within the human *Cox-2* promoter at −580 and −358. Supporting this mechanism, we found that E_2 significantly activated both 0.8 and 0.4 kb *Cox-2* promoters driving luciferase reporter constructs, suggesting that the NFκB binding site at −358 in particular is important.[3] Previous studies indicate that PI3K/Akt can regulate Cox-2 mRNA production or stability in positive or negative fashion, dependent upon the stimulus and cellular context (51, 52).

Cox-2 Protein Synthesis and Prostaglandin Secretion Are Stimulated by E_2—Cox-2 protein synthesis was then determined by Western blot. After 1-h exposure of EC to E_2, the

[3] A. Pedram, M. Razandi, M. Aitkenhead, C. C. W. Hughes, and E. R. Levin, unpublished observations.

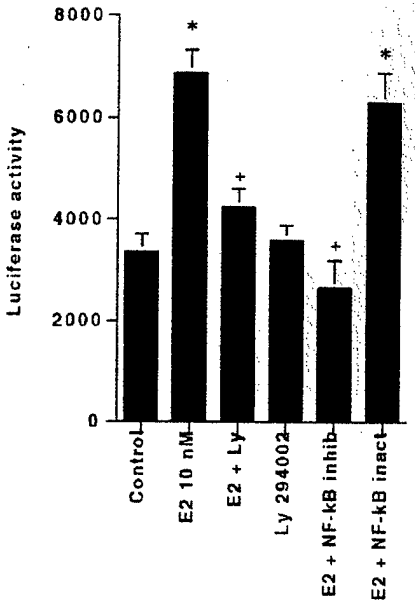


FIG. 4. **E_2 stimulates the activity of a *Cox-2* promoter/luciferase reporter via PI3K in endothelial cells.** EC were transfected with a plasmid to express a 1.8-kb *Cox-2* promoter plasmid driving the luciferase reporter. The cells were recovered and synchronized and then incubated for 6 h with 10 nM E_2 with or without 10 μM LY294002, or with 20 μM of either an NFκB active inhibitor(SN50M) or an inactive analog (SN50). The *bar graph* reflects the mean ± S.E. from triplicate determinations in each of two experiments, combined for analysis. *, $p < 0.05$ for control *versus* E_2; +, $p < 0.05$ for E_2 *versus* E_2 +LY294002 by analysis of variance plus Scheffe's test.

Cox-2 protein was nearly 3-fold increased in relevant E_2 concentration-responsive fashion (Fig. 5*A*). The PI3K inhibitors, LY 294002 and wortmannin, each caused an 80% reduction in Cox-2 protein synthesis, as did ICI 182,780, an ER antagonist. Expression of dominant negative constructs for the p85 subunit of PI3K (DN-PI3K) or Akt (DN-Akt) also resulted in substantial inhibition of E_2-induced Cox-2 protein expression. The dominant negative constructs had no effects alone (data not shown).

In preliminary studies, we determined a time course for PGE_2 and PGI_2 secretion in response to E_2. Compared with basal secretion (0 time), E_2 stimulated an initial 2-fold PGE_2 release at 10 min (first point assessed), reaching a maximum increase at 30 min and plateauing thereafter (data not shown). Based upon these results, BAEC were then incubated with 10 nM E_2 with or without inhibitors of ER, PI3K, and Cox-1 or Cox-2 for 30 min. As seen in Fig. 5*B*, E_2 stimulated a 13-fold increase of PGE_2 secretion, 75% prevented by ICI 182,780. Co-incubation of the cells with NS-398 (a specific Cox-2 inhibitor) reversed the E_2 effect by 86%. A specific Cox-1 inhibitor (SC-560) also provided a 33% inhibition of the E_2 effect, suggesting that PGE_2 synthesis was mainly dependent upon the Cox-2 enzyme. It is recognized that PGE_2 and PGI_2 can both result from either Cox-1 or Cox-2 enzymatic action, but in a given cell type or in response to a specific stimulus, one cyclooxygenase activity may predominate over the other (53). Importantly, the PI3K inhibitors, wortmannin and LY 294002 each prevented E_2-induced PGE_2 secretion by 87%. This is consistent with the ability of E_2 to activate the *Cox-2* gene in a PI3K-dependent fashion, as identified by microarray and reporter studies. Interestingly, the PGE_2 receptor was also found in our array to be up-regulated by E_2 via PI3K (Table I).

As for PGI_2, E_2 induced a 4-fold increase in secretion (Fig. 5*B*). This was 60% reversed by ICI 182,780, 58% by the Cox-2 inhibitor, and 36% by the Cox-1 inhibitor. The PI3K inhibitors also reduced the E_2 induction of PGI_2 by 60%. Our results

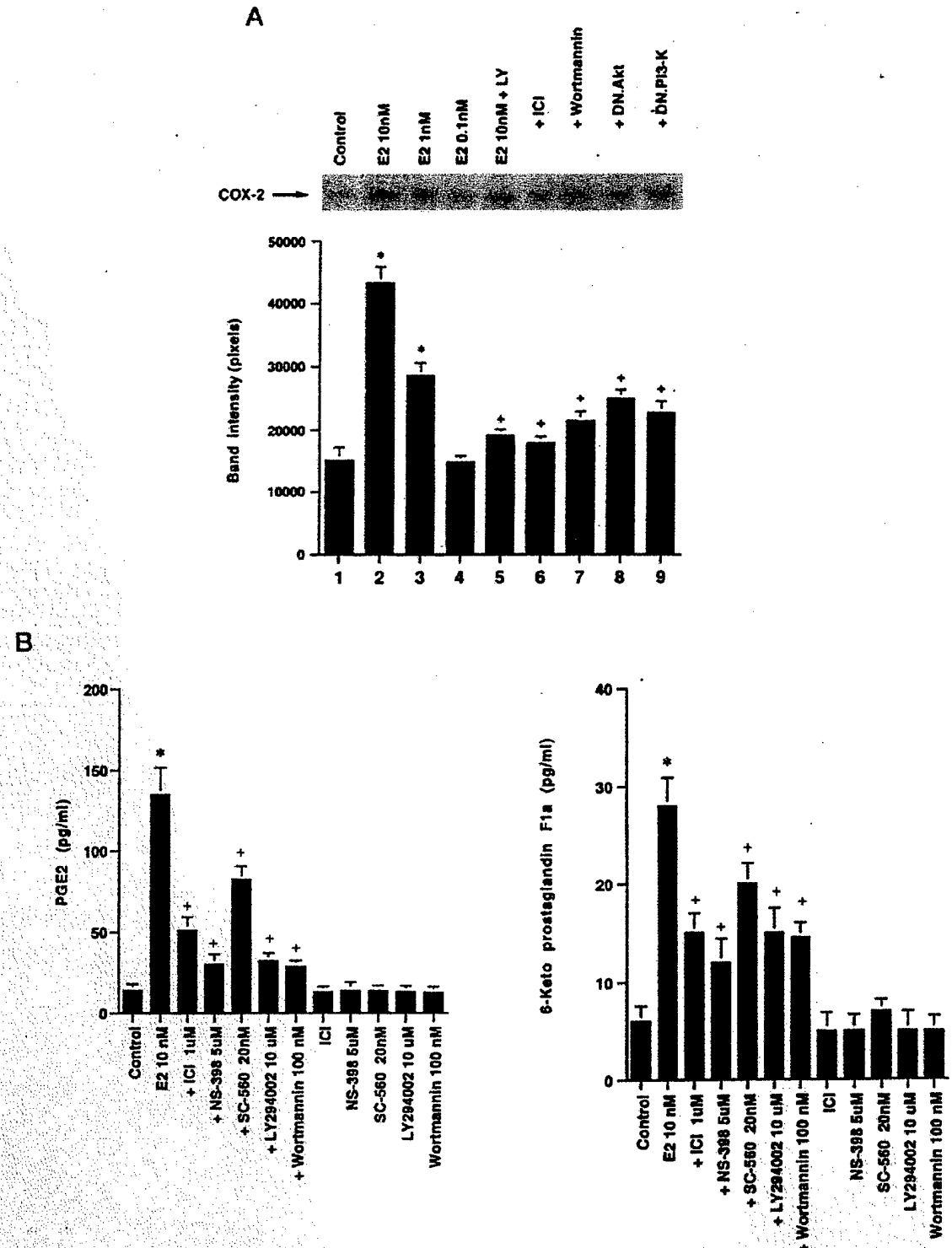
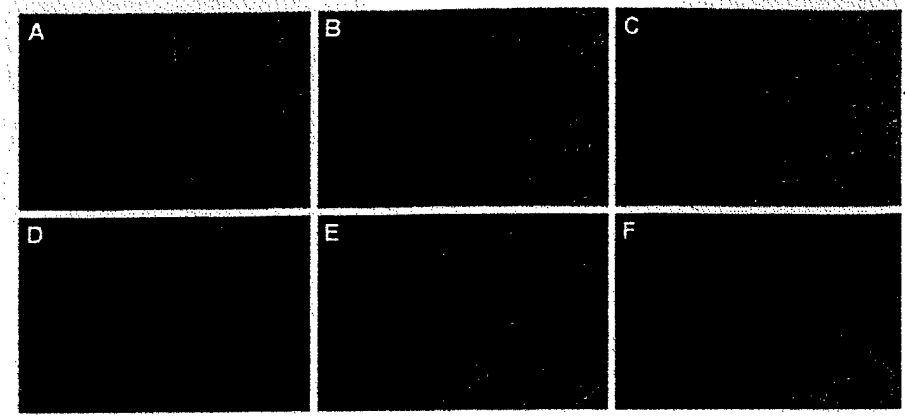


FIG. 5. *A*, E_2 stimulates the production of Cox-2 protein in EC via PI3K and Akt signaling. EC were incubated with several concentrations of E_2 for 60 min, and Cox-2 protein was determined by Western blot of immunoprecipitated cell lysate. In some conditions, LY294002 or ICI182780 was added 30 min prior to 10 nM E_2, or the cells were first transfected to express pMT2-AH-Akt (*DN.Akt*) or pcDNA3-delta p85 (*DN.PI3-K*). Control and E_2 with or without LY conditions were carried out in cells transfected with pcDNA3. A representative study is shown, and the *bar graph* reflects three experiments combined. *, $p < 0.05$ for control *versus* E_2; +, $p < 0.05$ for 10 nM E_2 *versus* E_2 plus inhibitor. *B*, secretion of PGE_2 (*left*) and PGI_2 (6-keto prostaglandin F1alpha) (*right*) in response to E_2. EC were incubated with 10 nM E_2 with or withoutPI3K inhibitors, or with E_2 + NS-398 (Cox-2 inhibitor) or SC-560 (Cox-1 inhibitor) for 30 min (soluble inhibitors were added 30 min prior to E_2). The *bar graph* represents three combined experiments.

greatly extend the observations of others that E_2 can rapidly stimulate the secretion of PGI_2 from endothelial cells (54, 55). Here we show transcriptional up-regulation of the *Cox-2* gene, increased protein production, and stimulation of both PGE_2 and PGI_2 secretion, in a PI3K-dependent fashion. An observation that is relevant for EC is that Cox-2 and cAMP-signaling enhances angiogenesis through the induction of vascular endothelial growth factor (56). Cox-2 and cAMP are up-regulated by



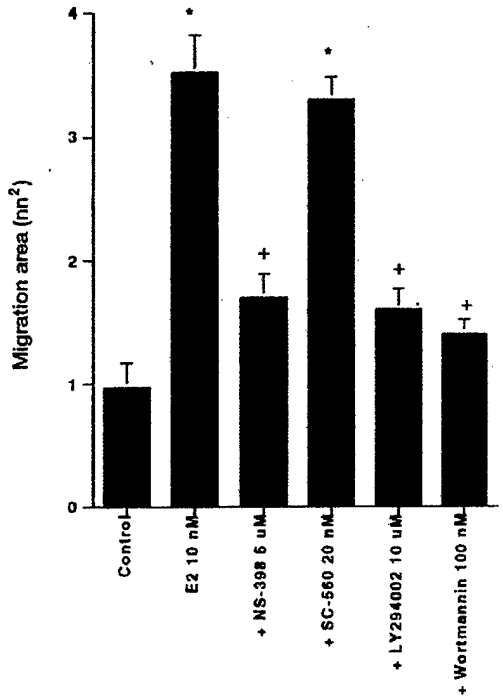


FIG. 6. **E_2 stimulates EC migration in PI3K and Cox-2 related fashion.** Cultured EC were cut with a surgical blade, and all cells were removed on the left side of the wound with a scraper. The remaining cells were then cultured overnight with/without 10 nM E_2 with or without PI3K and Cox-2 inhibitors. *Panel A* is control cells (no E_2 or serum); *panel B* is E_2. *Panel C* is E_2 + NS-398, *panel D* is E_2 + SC-560, *panel E* is E_2 + LY294002, and *panel F* is E_2 + wortmannin (50 nM). The inhibitors alone had no effects (data not shown). The *bar graph* below the composite reflects three experiments combined. *, $p < 0.05$ for control *versus* E_2; +, $p < 0.05$ for E_2 *versus* E_2 plus inhibitor.

E_2 signaling from the membrane (2, 6, and here). This may be significant to E_2-modulated induction and developmental function of vascular endothelial growth factor, in the formation and permeability of the blood vessels of the ovary and uterus (56, 57).

EC Migration—We then examined possible roles for Cox-2-derived prostaglandins and E_2 in mediating EC migration. E_2 is known to induce several aspects of angiogenesis, including EC migration (14), and Cox-2 also importantly participates in these processes (28). Cultured EC were "wounded," and the migration of EC across this wound barrier was determined after 24-h exposure to various conditions. As seen in Fig. 6, 10 nM E_2 (*panel B*) induced a 3-fold increase in migration area compared with control EC (*panel A*). The effect of E_2 was reduced 75% by a Cox-2 inhibitor (*panel C*), but not significantly by a Cox-1 inhibitor (*panel D*). The effect of E_2 was also prevented by LY294002 (*panel E*) and by wortmannin (PI3K inhibitor) (*panel F*). The inhibitors by themselves had no effects on EC migration (data not shown). Thus, we link the Cox-2 up-regulation in EC, induced by E_2 and PI3K signaling, to a cell biological outcome.

Increasingly, the integration of membrane and nuclear actions of steroids is recognized. An important mechanism demonstrated here is that steroids rapidly induce through kinase signaling many genes coding for transcription factors. We determined that the identified transcription factor gene promoters usually lack any form of estrogen response elements. Transcription factor up-regulation presumably then leads to the induction of additional genes that regulate steroid-induced cellular function. However, we also show that, by the same signaling mechanism, steroids rapidly up-regulate genes coding for enzymes or signaling molecules. The protein products of these genes both directly impact cell functions (*i.e.* cell migration) and induce additional transcription. Through membrane-initiated steroid signaling (MISS), we propose that estrogen affects overall cellular processes by post-translationally modulating the functions of existing proteins (9, 14) and by activating discrete programs of gene expression.

REFERENCES

1. Truss, M., and Beato, M. (1993) *Endocrine Rev.* **14**, 459–479
2. Aronica, S. M., Kraus, W. L., and Katznellenbogen, B. S. (1994) *Proc. Natl. Acad. Sci.* **91**, 8517–8521

3. Tesarik, J., and Mendoza, C. (1995) *J. Clin. Endocrinol. Metab.* **80,** 1438–1443
4. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997) *J. Biol. Chem.* **272,** 11902–11907
5. Pietras, R., and Szego, C. M. (1977) *Nature* **265,** 69–72
6. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13,** 307–319
7. Wyckoff, M. H., Chambliss, K. L., Mineo, C., Yuhanna, I. S., Mendelsohn, M. E., Mumby, S. M., and Shaul, P. W. (2001) *J. Biol. Chem.* **276,** 27071–27076
8. Singer, C. A., Figueroa-Masot, X. A., Batchelor, R. H., and Dorsa, D. M. (1999) *J. Neurosci.* **19,** 2455–2463
9. Razandi, M., Pedram, A., and Levin, E. R. (2000) *Mol. Endocrinol.* **14,** 1434–1447
10. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) *EMBO J.* **15,** 1292–1300
11. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M. E., and Shaul, P. W. (1999) *J. Clin. Invest.* **103,** 401–406
12. Kumar, R., Wang, E.-A., Mazumdar, A., Talukder, A. H., Mandal, M., Yang, Z., Bagheri-Yarmand, R., Sahin, A., Hortobagyi, G., Adam, L., Barnes, C. J., and Vadlamudi, R. K. (2002) *Nature* **418,** 654–657
13. Levin, E. R. (2001) *J. Appl. Physiol.* **91,** 1860–1867
14. Razandi, M., Pedram, A., and Levin, E. R. (2000) *J. Biol. Chem.* **275,** 38540–38546
15. Watters, J. J., Campbell, J. S., Cunningham, M. J., Krebs, E. G., and Dorsa, D. M. (1997) *Endocrinology* **138,** 4030–4033
16. Duan, R., Xie, W., Li, X., McDougal, A., and Safe, S. (2002) *Biochem. Biophys. Res. Commun.* **294,** 384–394
17. Duan, R., Xie, W., Burghardt, R. C., and Safe, S. (2001) *J. Biol. Chem.* **276,** 11590–11598
18. Porter, W., Saville, B., Hoivik, D., and Safe, S. (1997) *Mol. Endocrinol.* **11,** 1569–1580
19. Watters, J. J., Chun, T. Y., Kim, Y. N., Bertics, P. J., and Gorski, J. (2000) *Mol. Endocrinol.* **14,** 1872–1881
20. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) *Nature* **407,** 538–541
21. Long, A. D., Mangalam, H. J., Chan, B. Y. P., Toller, L., Hatfield, G. W., and Baldi, P. (2001) *J. Biol. Chem.* **276,** 19937–19944
22. Sheng, H., Shao, J., Dixon, D. A., Williams, C. S., Prescott, S. M., DuBois, R. N., and Beauchamp, R. D. (2000) *J. Biol. Chem.* **275,** 6628–6635
23. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) *EMBO J.* **16,** 2783–2793
24. Kong, M., Mounier, C., Wu, J., and Posner, B. I. (2000) *J. Biol. Chem.* **275,** 36035–36042
25. Pedram, A., Razandi, M., and Levin, E. R. (September 3, 2002) *J. Biol. Chem.* 10.1074/jbc.M202391200
26. Hyder, S. M., Nawaz, Z., Chiapetta, C., Yokoyama, K., and Stancel, G. M. (1995) *J. Biol. Chem.* **270,** 8506–8513
27. Lazennec, G., Alcorn, J. L., and Katzenellenbogern, B. S. (1999) *Mol. Endocrinol.* **13,** 969–980
28. Matsumoto, H., Ma, W.-g., Daikoku, T., Zhao, X., Paria, B. C., Das, S. K., Trzaskos, J. M., and Dey, S. (2002) *J. Biol. Chem.* **277,** 29260–29267
29. Locklin, R. M., Riggs, B. L., Hicok, K. C., Horton, H. F., Byrne, M. C., and Khosla, S. (2001) *J. Bone Miner. Res.* **16,** 2192–2204
30. Plant, A., Samuels, A., Perry, M. J., Colley, S., Gibson, R., and Tobias, J. H. (2002) *J. Cell Biochem.* **84,** 285–294
31. Watanabe, Y., Kawakami, K., Hirayama, Y., and Nagano, K. (1993) *J. Biochem.* **114,** 849–855
32. Dzurba, A., Ziegelhoffer, A., Vrbjar, N., Styk, J., and Slezak, J. (1997) *Mol. Cell Biochem.* **176,** 113–118
33. Schafer, U., Schneider, A., and Neugebauer, E. (2000) *Biochim. Biophys. Acta* **1494,** 269–276
34. Khachigan, L. M., Lindner, V., Williams, A. J., and Collins, T. (1996) *Science* **271,** 1427–1431
35. Du, B., Kent, K. C., Bush, H., Jr., Schulick, A. H., Kreiger, K., Collins, T., and McCaffrey, T. A. (2000) *J. Biol. Chem.* **275,** 39039–39047
36. Mendelsohn, M. E., and Karas, R. H. (1999) *New Engl. J. Med.* **340,** 1801–1811
37. Richards, J. S., Sharma, S. C., Falender, A. E., and Lo, Y. H. (2002) *Mol. Endocrinol.* **16,** 580–599
38. Tsai, K. J., Chen, S. K., Ma, Y. L., Hsu, W. L., and Lee, E. H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 3990–3995
39. Faletti, C. J., Perrotti, N., Taylor, S. I., and Blazer-Yost, B. L. (2002) *Am. J. Physiol.* **282,** C494–C500
40. Sherwin, B. B. (1998) *Proc. Soc. Exp. Biol. Med.* **217,** 17–22
41. Ediger, T. R., Kraus, W. L., Weinman, E. J., and Katznellenbogen, B. S. (1999) *Endocrinology* **140,** 2976–2982
42. Kane, L. P., Mollenauer, M. N., Xu, Z., Turck, C. W., and Weiss, A. (2002) *Mol. Cell. Biol.* **16,** 5662–5674
43. Tsai, M. S., Bogart, D. F., Li, P., Mehmi, I., and Lupu, R. (2002) *Oncogene* **21,** 964–973
44. Heany, A. P., Fernando, M., and Melmed, S. (2002) *J. Clin. Invest.* **109,** 277–283
45. Lederman, R. J., Mendensohn, F. O., Anderson, R. D., Saucedo, J. F., Tenaglia, A. N., Hermiller, J. B., Hillegass, W. B., Rocha-Singh, K., Moon, T. E., Whitehouse, M. J., Annex, B. H., and TRAFFIC investigators. (2002) *Lancet* **359,** 2048–2050
46. Ribardo, D. A., Kuhl, K. R., Peterson, J. W., and Chopra, A. K. (2002) *Toxicon* **40,** 519–526
47. Li, D. Y., Hardy, P., Abran, D., Martinez-Bermudez, A. K., Guerguerian, A. M., Bhattacharaya, M., Almazan, G., Peri, K. G., Varma, D. R., and Chemtob, S. (1997) *Am. J. Physiol.* **273,** R1283–R1290
48. Hocheri, K., Endemann, D., Kammerl, M. C., Grobecker, H. F., and Kurtz, A. (2002) *Br. J. Pharmacol.* **136,** 1117–1126
49. Pandey, S. K., He, H. J., Chesley, A., Juhaszova, M., Crow, M. T., and Bernier, M. (2002) *Endocrinology* **143,** 375–385
50. Koul, D., Yao, Y., Abbruzzese, J. L., Yung, W. K., and Reddy, S. A. (2001) *J. Biol. Chem.* **276,** 11402–11408
51. Monick, M. M., Robeff, P. K., Butler, N. S., Flaherty, D. M., Carter, A. B., Peterson, M. W., and Hunninghake, G. W. (2002) *J. Biol. Chem.* **277,** 32992–33000
52. Tang, Q., Chen, W., Gonzales, M. S., Finch, J., Inoue, H., and Bowden, G. T. (2001) *Oncogene* **20,** 5164–5172
53. Coceani, F., Ackerley, C., Seidlitz, E., and Kelsey, L. (2001) *Br. J. Pharmacol.* **132,** 241–251
54. Akarasereenont, P., Techatraisak, K., Thaworn, A., and Chotewuttakorn, S. (2000) *Inflamm. Res.* **49,** 460–465
55. Shareman, T. S., Chambliss, K. L., Gibson, L. L., Pace, M. C., Mendelsohn, M. E., Pfister, S. L., and Shaul, P. W. (2002) *Am. J. Respir. Cell Mol. Biol.* **26,** 610–616
56. Amano, H., Hayashi, I., Yoshida, S., Yoshimura, H., and Majima, M. (2002) *Hum. Cell* **15,** 13–24
57. Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H. P., Nguyen, T. N., Peers, D., Chislom, V., Hillan, K. J., and Schwall, R. H. (1998) *Nat. Med.* **4,** 336–340

membrane ER acts similarly to many other GPCR that also lack catalytic or kinase domains yet signal to important events in cell biology.

As a GPCR, the membrane ER associates with and activates several G proteins. In transfected CHO cells, membrane ERα or ERβ co-precipitates with and activates $G\alpha_s$ and $G\alpha_q$ proteins (6). This leads to the expected downstream signaling to cAMP and inositol 1,4,5-trisphosphate generation, signaling that has been shown in cells expressing endogenous ER (22, 23). In EC, endogenous membrane ER physically associates with $G\alpha_i$ and activate endothelial nitric-oxide synthase; this probably takes place within caveolae (7). Additionally, it has been proposed in breast cancer cells that E2/ER transactivates the epidermal growth factor receptor (EGFR), leading to the downstream signaling to ERK activation (24, 25). This occurs through the activation of Gβγ, the liberation of heparin-binding EGF (HB-EGF), which results in the binding and activation of the EGFR, and the subsequent stimulation of the ERK signaling cascade. In some of these respects, the membrane ER acts similarly to a wide range of GPCR (26). However, it was further proposed in breast cancer cells that E2 in some undefined way activates the orphan GPCR, GPR30, to stimulate signaling, and this interaction does not require ER (25). These latter data are not in concert with many studies from other laboratories, indicating that E2 requires an ER for signaling from the membrane in various cell types (5, 6, 8, 20, 27, 28).

The utilization of EGFR by E2/ER to signal results from a linked series of events involving multiple upstream molecules, only some of which have been defined. For instance, we do not know the range of G proteins that can be activated to cross-talk to EGFR activation, and it is not clear what signals immediately downstream of G proteins are important. Src participates in the transactivation of EGFR in response to other GPCR ligands and is probably upstream of HB-EGF shedding (29), but its exact role and requirement for ER signaling is unclear. Furthermore, although matrix metalloproteinase (MMP) activation is required for HB-EGF liberation (and subsequent EGFR activation), the identity of the required MMP(s) is mainly undefined, especially as regards ER signaling. These issues are addressed in the studies described here. Finally, much of the interaction between GPCRs and EGFR has examined ERK activation. Thus, we sought additional signaling molecules in several cell types and the structural requirements within ER that utilize this interactive mechanism following endogenous ER ligation by E2.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and substrate for kinase activation/activity were from Santa Cruz Biotechnology (Santa Cruz, CA). PD 98059 was a generous gift from Dr. Alan Saltiel (Parke-Davis). LipofectAMINE was from Invitrogen. Primary cultures of bovine aortic EC were prepared and used as previously described (30). In transfection studies, EC were generally used in passages 4 and 5, based upon the previous observation that this greatly increases the transfection efficiency of these cells. Breast cancer cell lines were obtained from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 or RPMI 1640 with serum until 48 h prior to experimentation, when they were placed in serum-free conditions and in medium without phenol red. Gelatin was from Sigma, and kinase substrates were from Upstate Biotechnology, Inc. (Lake Placid, NY) or Santa Cruz Biotechnology. PP2, Src family kinase inhibitor, and GM6001, a matrix metalloproteinase (MMP) inhibitor, were from Calbiochem (San Diego, CA).

Kinase Activity Assays—For ERK or p38β activity assays, the cells were synchronized for 24 h in serum- and growth factor-free medium. The cells were then exposed to E2 for 8 (ERK) or 15 (p38) minutes, with or without additional substances, as previously described (30, 33). The cells were lysed, and lysate was immunoprecipitated with protein A-Sepharose conjugated to antiserum for p38 or ERK. Immunoprecipitated kinases were washed and then added to the proteins ATF-2 (for p38) or myelin basic protein (for ERK) for *in vitro* kinase assays. This was followed by SDS-PAGE separation and autoradiography/laser densitometry. In addition, the E2-induced phosphorylation of AKT kinases at 10 min was determined to assess activation. Cultured cell lysates were pelleted and dissolved in SDS sample buffer, boiled, separated, and then transferred to nitrocellulose. Phosphorylated kinase proteins were detected using phospho-specific monoclonal antibodies (Santa Cruz) and the ECL Western blot kit (Amersham Biosciences). Equal samples from the cells were also immunoprecipitated, and immunoblots of the precipitated kinase protein from each experimental condition were determined to show equal gel loading. All of the experiments were repeated two or three times.

Transient Transfections—MCF-7, HCC-1569, ZR-75-1, or bovine aortic endothelial cells (passages 4 and 5) were grown to 40–50% confluence and then transiently transfected with 1.5 μg (each well of 6-well plates) or 10 μg of fusion plasmid DNA (100-mm dishes). Plasmids included wild type mouse ERα (31) (kindly provided by Dr. Ken Korach) PRK5-HER, a dominant negative EGF receptor construct (kindly provided by Dr. A. Ullrich (32), a dominant negative Src construct, pRC-csrc-K298M (kindly provided by Drs. Louis Luttrell and Robert Lefkowitz (26), a dominant negative, truncated β-adrenergic receptor kinase plasmid (BARK1-CT pRK5) from Dr. Walter Koch (34), and truncated Gα subunit plasmids, serving as specific dominant negative constructs for G_s, G_q, G_i, G_{12}, and G_{13} (35). Transfection was carried out using LipofectAMINE (Invitrogen). The cells were incubated with liposome-DNA complexes at 37 °C for 5 h, followed by overnight recovery in culture medium containing 10% fetal bovine serum, 24 h of synchronization in serum-free medium, and then treatment with E2 with or without other substances.

Gelatin Zymography Substrate Cleavage and Antisense Studies for MMP Activity—MMP activity, as secreted into the medium of cultured MCF-7 cells, was analyzed by substrate gel electrophoresis (zymography). The cells were synchronized in serum-free medium for 24 h and then incubated in medium with or without 10 nM estrogen for 2 min at 37 °C in a CO_2 incubator. The cell medium was removed, concentrated 20-fold by ultrafiltration, and mixed with native gel sample buffer (Bio-Rad), and the proteins were separated by electrophoresis on an 8% gel co-polymerized with 1 mg/ml gelatin (Sigma). Active MMP-2 and MMP-9 (Calbiochem) was loaded into additional lanes on the gel. After electrophoresis, the gels were washed in 2.5% Triton X-100 at room temperature for 1 h and incubated for16 h at 37 °C (in 0.05 M Tris, pH 7.5, 5 mM $CaCl_2$, 0.02% NaN_3). The gel was stained with 0.5% Coomassie Blue and destained in 10% acetic acid, 10% propanol. The study was repeated twice. Gelatinolytic activity appears as a clear band on a blue background. For the fluorescent substrate assay, MCF-7 cells were synchronized for 24 h and then incubated without or with 10 nM estrogen for 2 min. The incubation medium was concentrated 10-fold, and 1 ml of assay buffer (100 mM Tris, pH 7.5, 100 nM $NaCl_2$) containing 5 μM of the Mca-Pro-Leu-Dpa-Ala-Arg-NH_2 substrate for MMP-2/MMP-9 was added and then incubated at 37 °C for 3 h. Excitation at 328 nm and emission at 393 nm were determined in a fluorimeter. To implicate MMP-2 and MMP-9 in the shedding of HB-EGF, the cells were incubated with antisense (ASO) or scrambled antisense (MSO) with the same base composition for each of the two MMPs. The oligonucleotides were: MMP-2, ASO, CCGGGCCATTAGCGCCTCCAT, and MSO, TCACCGCGGTACGCATGCCCT; and MMP-9, ASO, CAGGGGCTGCCAGAGGCTCAT, and MSO, GCGAGCTAGGACTGTGCAGCC. The oligonucleotides were added with LipofectAMINE for 5 h, and the cells were recovered overnight and synchronized in the absence of serum for 12 h. Transfection efficiency exceeded 60%, based upon co-expression of PEGFPc2. Western blot studies were carried out to confirm the efficacy of the ASO but not the MSO to inhibit specific protein production. Studies of E2-induced signaling were then carried out in cells expressing the various oligonucleotides.

Western Blot for HB-EGF and EGFR Phosphorylation—Subconfluent, transfected, or nontransfected cultured bovine aortic endothelial cells were serum-deprived for 24 h and then incubated under various conditions for 10 min with inhibitors followed by 10 min of treatment with stimulants. This included several 17-β-E2 concentrations, ICI 182780 (1 μM), and 100 nM GM6001, a broad MMP inhibitor. The cells were lysed, and antibodies to HB-EGF or EGFR (tyrosine 1138) (1:50 dilution) were conjugated to Sepharose beads and then added to the cell lysate for 2 h at 4 °C. After pelleting and washing, the samples were electrophoretically separated on a 7% SDS gel, transferred to nitrocellulose, and immunoblotted. Detection utilized the ECL kit (Amersham Biosciences).

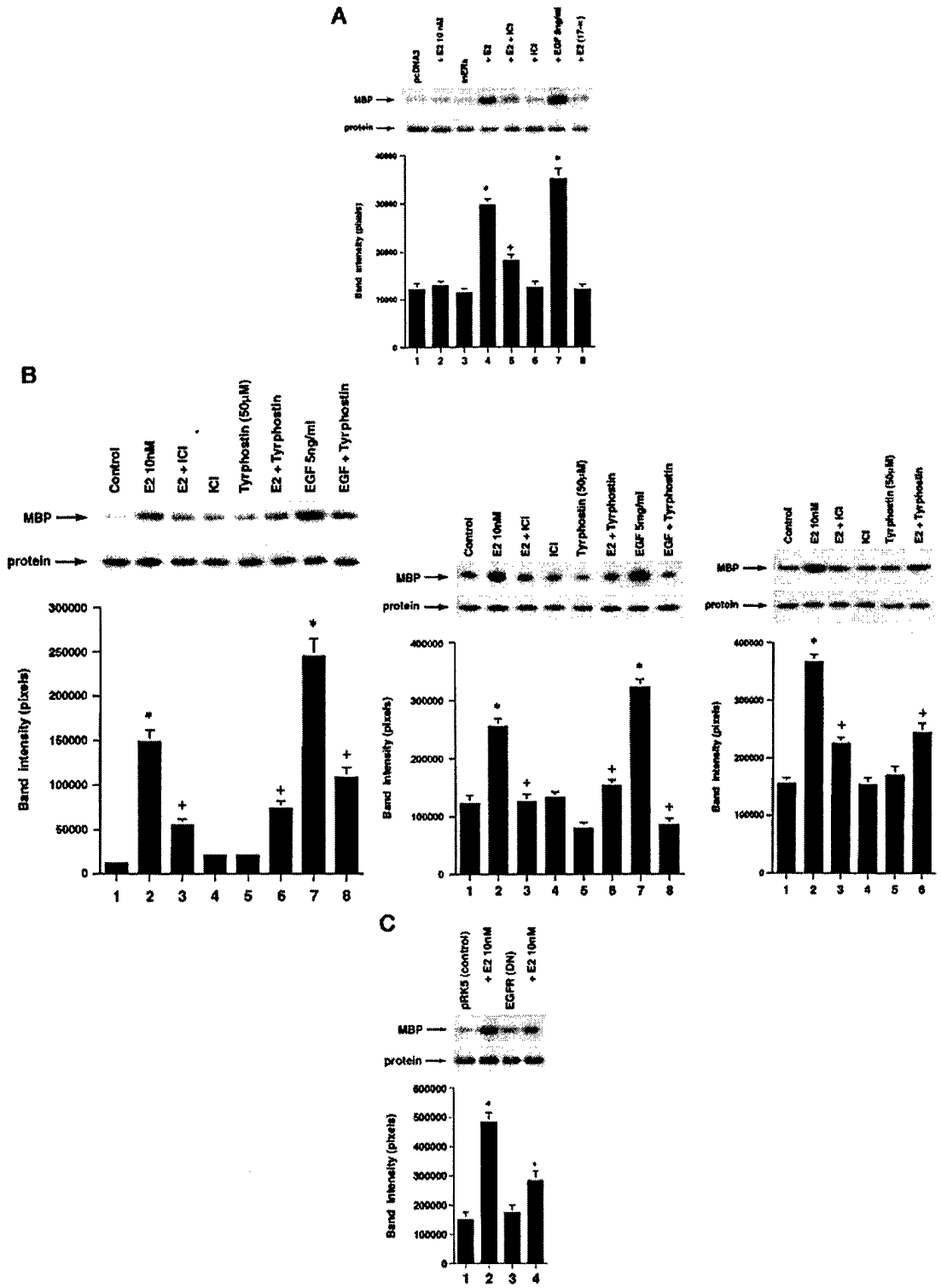


FIG. 1. **E2 activates ERK via ER and EGFR.** *A*, 17-β-E2 activates ERK only when ER is present. HCC-1569 cells (ER negative) were incubated with 10 nM 17-β-E2 or were transfected to express wild type mouse ERα (*mERa*) and then incubated with 17-β-E2 or 17-α-E2, and ERK activity (against myelin basic protein) was determined after 8 min in an *in vitro* tube assay as described under "Experimental Procedures." Immunoblots of total ERK protein are shown below the activity. The *bar graph* represents three combined experiments. *, $p < 0.05$ for control *versus* E2 or EGF; +, $p < 0.05$ for E2 *versus* E2 with ICI182780 (ER antagonist). *B*, inhibition of EGFR tyrosine kinase function with tyrphostin AG1478 prevents E2-induced ERK activation in MCF-7 cells (*left panel*), in ZR-75–1 cells (*center panel*), or in endothelial cells (*right panel*). The cells were incubated as described above with 17-β-E2 with or without a specific EGFR tyrosine kinase inhibitor, and ERK activity was determined. Each *bar graph* represents three combined experiments. *, $p < 0.05$ for control *versus* E2 or EGF; +, $p < 0.05$ for E2 *versus* E2 with ICI182780 (ER antagonist) or E2 or EGF *versus* either E2 or EGF with AG1478 (tyrphostin). *C*, expression of a dominant negative EGFR (*EGFR (DN)*) prevents E2-induced ERK activation in MCF-7 cells. The cells were transfected to transiently express PRK5-HER dominant negative EGFR, recovered overnight in 10% serum, and 24 h after cell recovery, E2 activation of ERK was determined after 8 min of incubation. *, $p < 0.05$ for control *versus* E2; +, $p < 0.05$ for E2 *versus* PRK5-HER transfected cells incubated with E2.

RESULTS

Activation of ERK by E2 Requires an ER and the Activation of EGFR by HB-EGF—We first established that E2 required both the presence of an ER and the activation of EGFR to signal to ERK. HCC-1569 cells lack ER, and the cells did not respond to E2 with ERK activation (Fig. 1*A*, *lanes 1* and *2*). When ERα was expressed in these cells, 17-β-E2 (*lane 4*), but not 17-α-E2 (*lane 8*), was capable of activating ERK, and this was substantially blocked by the ER antagonist, ICI182780 (*lane 5*). As a positive control, these cells express the EGFR and appropriately respond to EGF (*lane 7*). The requirement of ER is similar to our previous findings in CHO-K1 cells (6). We then asked whether E2 activation of ERK depends upon EGFR tyrosine kinase activity. We examined this in MCF-7 and ZR-75–1 breast cancer cells and EC (all with ER). Tyrphostin AG1478, specifically directed against the EGFR tyrosine kinase function, prevented EGFR-induced ERK activation in both MCF-7 and ZR-75–1 cells (Fig. 1*B*, *left* and *center panels*). Importantly, tyrphostin AG1478 also substantially prevented the ability of E2 to activate ERK in the three cell types (Fig. 1*B*, *all panels*, *lanes 2 versus lanes 6*). To corroborate this finding, we expressed a dominant negative EGFR (31) in MCF-7 cells, and E2 was much less effective in stimulating this MAP kinase, compared with cells expressing the empty vector (control) (Fig. 1*C*).

What ligand for EGFR is involved in the transactivation of this receptor by E2? Although there are many members of the EGF family that can bind the EGFR, HB-EGF has often been implicated in the setting of GPCR signaling via this receptor (36). To examine this, we first determined whether E2 could stimulate the secretion of HB-EGF, determined by Western blot. As seen in Fig. 2*A*, E2 dose-responsively induced a significant enhancement of HB-EGF shedding/secretion from the MCF-7 cells after 3 min of incubation. This was prevented by ICI182780 and by GM6001, an MMP inhibitor. To determine that HB-EGF was the important ligand for EGFR signaling to ERK, we incubated the MCF-7 cells with 10 nM E2, in the presence or absence of antibody to HB-EGF. In the setting of this added antibody, E2 could not significantly activate ERK (Fig. 2*B*). In contrast, antibody to TGFα-1, another ligand for the EGFR, had no effect on E2-induced ERK, and the antibodies by themselves did not affect basal ERK activity. Similarly, antibody to HB-EGF (but not to TGFα-1) prevented E2-induced phosphorylation of the EGFR (Fig. 2*C*). Identical findings were determined from EC incubated with E2 (data not shown). These results support the interactions of secreted HB-EGF with EGFR, leading to ERK activation in breast cancer and vascular cells. The data also support ER-mediated, MMP-dependent release of HB-EGF.

Matrix Metalloproteinases 2 and 9 Are Activated and Are Necessary for Signaling by E2—Current evidence supports the idea that GPCRs activate MMP activity, thereby liberating HB-EGF from the cell matrix, leading to the transactivation of the EGFR (36, 37). Therefore, MMP activation represents the step immediately upstream of HB-EGF liberation. In many cell paradigms, including E2 action, the precise MMP(s) activated by GPCR signaling are unknown. We therefore showed that E2 activates MMP activity by demonstrating that the incubation medium from MCF-7 cells treated with E2 for 2 min induces the cleavage of substrate specific for MMP-2 and MMP-9 (Fig. 3*A*). In contrast, substrate specific for MMP-13 or MMP-3 was not cleaved by the E2-treated cell medium (data not shown), even though breast cancer cells produce these proteolytic enzymes. We then sought to further identify the MMPs by carrying out gelatin zymography. E2 treatment of the cultured MCF-7 cells for 2 min led to the increased secretion and activation of MMP-2 and -9 (Fig. 3*B*, *first* and *second lanes*). To corroborate the identify of the digested gelatin band activities, active MMPs (Calbiochem) were also run in parallel on a separate gel (data not shown). Functionally, activation of MMP activity was necessary for E2-induced ERK. This was shown in that the MMP inhibitor completely reversed the ability of E2 to activate ERK in both MCF-7 and ZR-75–1 cells (Fig. 3*C*, *left* and *right panels*). This compound did not affect EGF-induced ERK activation, supporting the idea that MMP-related events occur upstream to EGFR activation in this pathway.

Although E2 activates these two MMPs, it is not clear that they are responsible for E2-induced HB-EGF shedding. We therefore used ASO or MSO, with the latter comprised of the same base composition as the ASO for MMP-2 and MMP-9, and expressed them in MCF-7 cells. First, we validated the constructs by showing that the ASO (but not the MSO) for MMP-2 or MMP-9 inhibited the respective protein production in a dose-related manner (Fig. 4*A*, *left panel*). Similarly, we validated the function of the MMP-2 or MMP-9 to specifically inhibit only the intended protein target (Fig. 4*A*, *right panel*). Using these ASO and MSO, we next determined whether MMP-2 and MMP-9 each contributed to HB-EGF shedding and ERK activation (Fig. 4, *B* and *C*). Each ASO significantly downregulated E2-induced HB-EGF liberation, and expressing the ASO to both MMPs completely blocked this E2 action. The ASO to MMP-2 almost completely prevented the ability of E2 to activate ERK in MCF-7 cells, whereas the ASO to MMP-9 was also substantially able to prevent this signaling; neither MSO had any effect, and the results were similar to those in EC. E2/ER stimulation of MMP-2 and MMP-9 may therefore underlie several important actions in breast cancer, including signaling through ERK to cell proliferation and survival (5, 9). Metalloproteinase activation also contributes to the disengagement of cells from matrix, a necessary initial step preceding invasion and migration behaviors (38). MMP-2 and MMP-9 are well recognized to contribute to these events in various contexts (38, 39).

Specific G Proteins Are Involved in E2-induced Transactivation of EGFR—It has previously been established that E2 can activate $G\alpha_s$ and $G\alpha_q$, as well as $G\alpha_i$ in several cell models (6, 7). Therefore, one or more G proteins activated by E2 could ultimately result in EGFR signaling to ERK. To examine this issue, we expressed mini-genes for Gα subunits of G_s, G_i, G_{12}, G_{13}, and G_q, constructs that have been shown to act as dominant negatives for specific endogenous G protein subunit activation (35). As seen in Fig. 5*A*, ERK activation in response to E2 in cells expressing the control plasmid, $G\alpha_{ir}$ (*lane 3*), was substantially prevented after expressing the inhibitory minigenes for $G\alpha_i$ and $G\alpha_q$ (*lanes 4* and 5). However, dominant negative constructs for the α subunits of G_s, G_{12}, and G_{13} had insignificant effects on this signaling. We also expressed a C-terminal truncated β-adrenergic receptor kinase, pRK5-BARK1-(495–689), that inhibits G$\beta\gamma$ signaling (33). Expression of this construct significantly but incompletely prevented the ability of E2 to activate ERK and HB-EGF liberation (Fig. 5*B*). Upon expressing ERα in HCC-1569 cells, E2 could now activate ERK in a $G\alpha_i$, $G\alpha_q$, and G$\beta\gamma$-dependent fashion (Fig. 5*C*). Therefore, both Gα and G$\beta\gamma$ subunits contribute to the ability of E2/ER to activate the signaling pathway that ultimately results in EGFR transactivation.

Calcium, PLC, and PKC Activities Mediate E2-induced MMP Activation—The signaling through the identified G proteins potentially leads to the activation of MMP activity and the subsequent downstream signaling through EGFR. We examined which signal pathways immediately downstream of G protein activation that we identified here could mediate MMP activation. E2-induced MMP activity was significantly inhib-

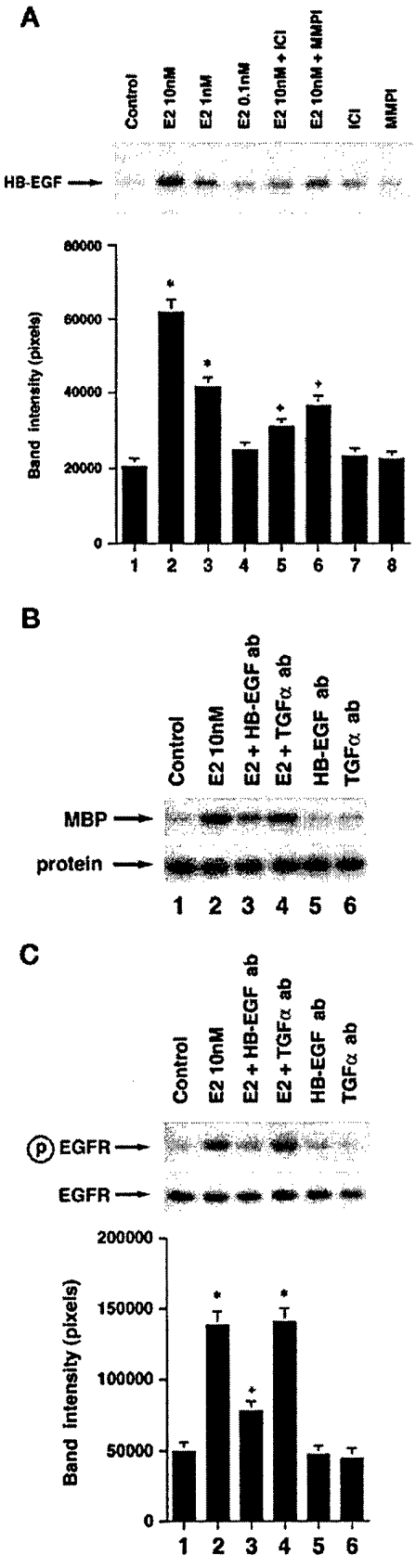


FIG. 2. **E2 rapidly stimulates HB-EGF release via matrix metalloproteinase action and ER.** *A*, MCF-7 cells were incubated with E2, 0.1–10 nM with or without ICI182780, or 100 nM GM6001, an MMP inhibitor, for 3 min. The HB-EGF shed into the medium was determined by Western blot. The *bar graph* is three experiments combined. *, $p < 0.05$ for control *versus* E2; +, $p < 0.05$ for E2. *B*, antibody to HB-EGF but not TGFα blocks E2-induced ERK activation. MCF-7 were incubated with 10 nM E2 with or without 10 μg/ml antibody to HB-EGF or TGFα, and ERK activity was determined after 8 min. The antibodies alone had no effect on ERK activity. *C*, HB-EGF but not TGFα antibody blocks the E2-induced transactivation/phosphorylation of EGFR. The cells were incubated with 10 nM E2 with or without antibodies for 8 min, and lysate was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody to tyrosine 1173 of the EGF receptor. *, $p < 0.05$ for control *versus* E2 or E2 plus TGFα antibody; +, $p < 0.05$ for E2 *versus* E2 with HB-EGF antibody.

ited by EDTA, an extracellular calcium chelator, but was not affected by BAPTA-AM (Fig. 2*A*). This indicates that calcium entry through surface channels, but not the mobilization of intracellular calcium, contributes to E2-induced MMP activation. It has previously been shown that E2 activates several calcium channels that lead to an influx of calcium into the cell (40, 41), and this can result from $G\alpha_q$ or $G\beta\gamma$ activation.

We also found that soluble inhibitors of PLC and PKC (calphostin C and U-73122, respectively) significantly prevented E2 activation of MMP activity (Fig. 3*A*). This is consistent with our identification here of $G\alpha_q$ and $G\beta\gamma$ as mediating E2-induced ERK activation, because PLC and PKC up-regulation results from the activation of these G protein subunits. We previously showed that E2 can activate $G\alpha_q$, PLC, and inositol 1,4,5-trisphosphate generation via membrane ER (6), and E2 has been described to stimulate PKC activity in several cell types (reviewed in Ref. 42). PKC-dependent signaling in growth plate chondrocytes mediates E2-induced regulation of these cells, and originates from membrane action of the steroid (43). These findings link the most proximal signaling events to later events (MMP activation and HB-EGF shedding), mediating EGFR transactivation.

Role of Src in Shedding of HB-EGF—It has been documented that E2-liganded ER complex with and activate the Src tyrosine kinase, and this is necessary for E2 stimulation of ERK (8, 17). Src could potentially play a role both upstream and downstream of EGFR activation. We therefore determined where Src activation is required for the proximal signaling induced by E2, leading to EGFR transactivation. As shown in Fig. 3*B*, E2-induced MMP-2 and MMP-9 activation and secretion at 2 min (*first lane versus second lane*). This was substantially prevented by the Src family kinase inhibitor, PP2 (*third lane*), or by expressing a specific dominant negative Src construct, pRC-csrc-K298M (26) (*fifth* and *sixth lanes* compared with *first* and *second lanes*). Thus, these results define a novel role for Src in E2-induced signaling from the membrane, and we suggest that this molecule may play a similar role in other GPCR-induced activation of EGFR through this mechanism.

ER Is Required for Proximal Signaling Events—We earlier showed that E2 requires an ER to activate ERK (Fig. 1). To further support the idea of the necessity of ER presence for E2 action, we expressed ERα in HCC-1569 cells and determined the proximal signaling events implicated. We first demonstrated that expression of the dominant negative $G\alpha_i$ and $G\alpha_q$ mini-genes substantially blocked E2-induced ERK, compared with kinase activity in the presence of the control (inactive) construct, $G\alpha_{ir}$ (Fig. 6*A*). Similarly, expression of C-terminal truncated β-adrenergic receptor kinase (BARK1) also down-regulated E2/ER-induced ERK, whereas the truncated mini-gene for $G\alpha_{13}$ was without effect (similar to control). We also examined HB-EGF secretion and found that E2 stimulated the secretion of this receptor ligand only when ER was expressed (Fig. 6*B*, *lanes 1* and *2 versus lanes 3* and *4*). ICI182780 and MMP inhibition significantly prevented the stimulation of HB-EGF secretion. Finally, we found that in the presence (but not

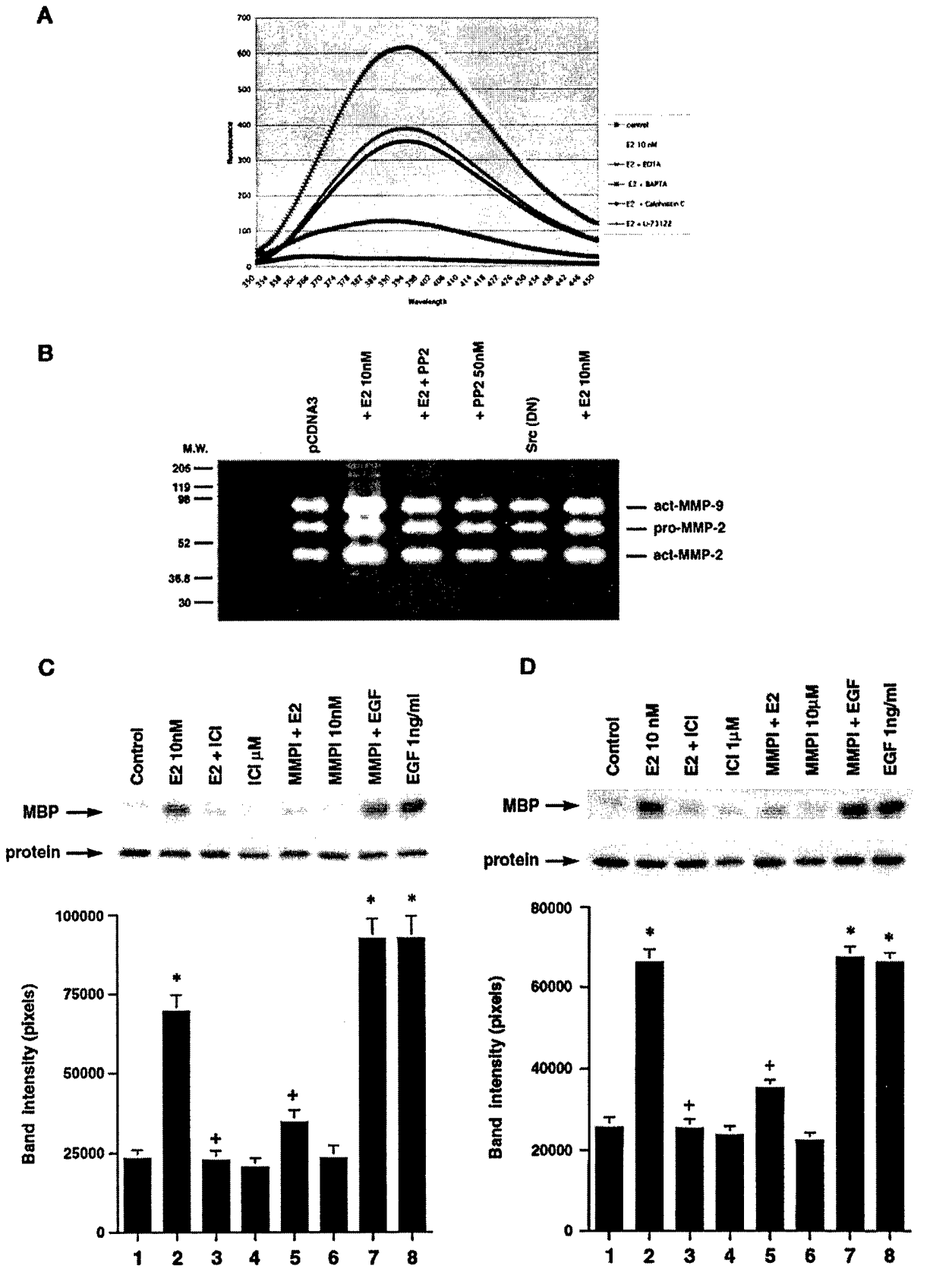


FIG. 3. **E2 activates matrix metalloproteinase 2 and 9 secretion and activity.** *A*, cells were incubated with or without E2 with or without BAPTA (intracellular calcium inhibitor), EDTA (chelates extracellular calcium), a specific PLC inhibitor, U73122, or a specific PKC inhibitor, calphostin C for 2 min. Cleavage of substrate for MMP-2/MMP-9 by the medium from MCF-7 cells incubated with 10 nM E2 for 2 min was determined by spectroflurometry. The data are from triplicate determinations in a representative experiment, repeated twice. *B*, MCF-7 cells were

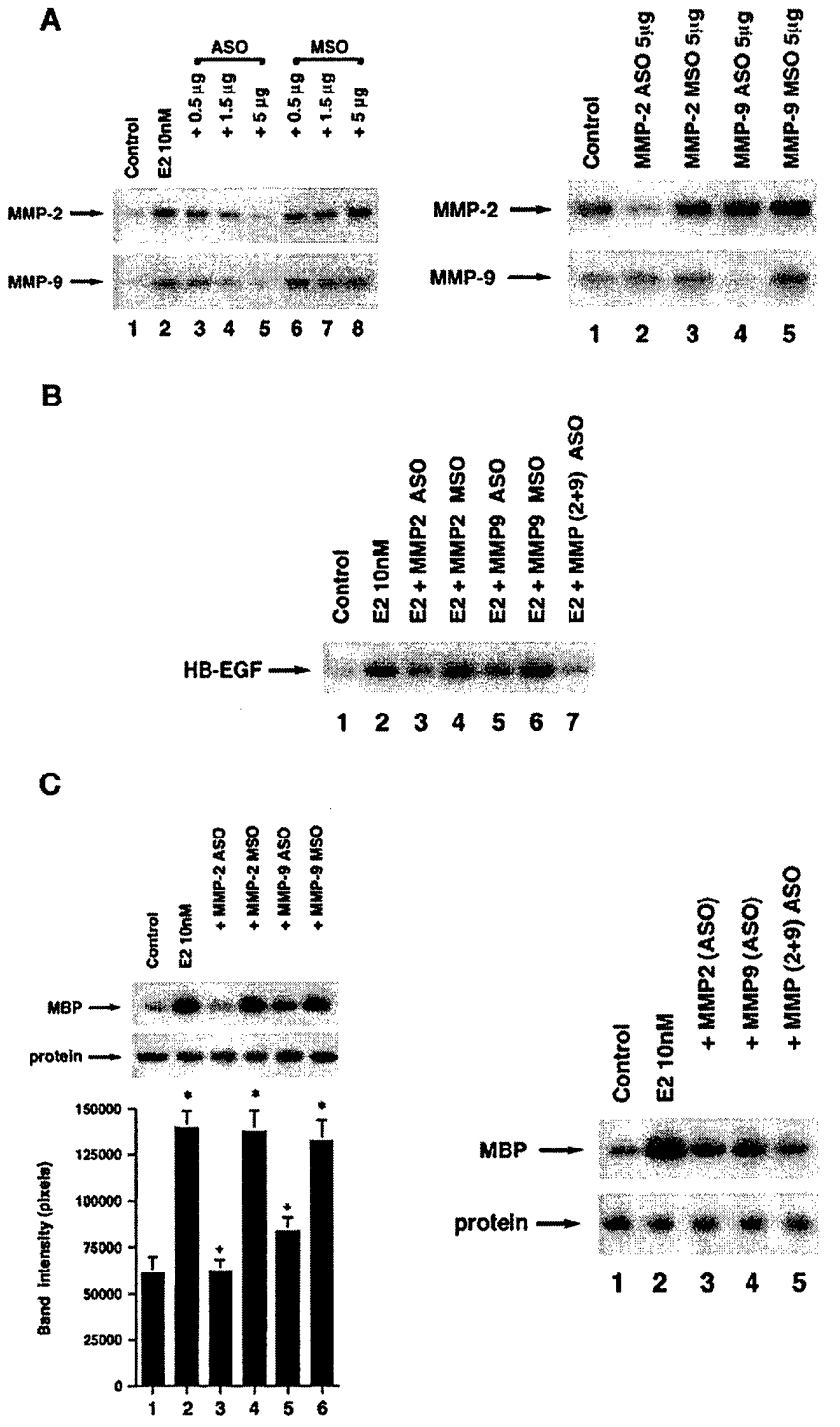


FIG. 4. **Matrix metalloproteinases 2 and 9 mediate E2/ER effects.** *A*, validation of ASO to MMP-2 and MMP-9. MCF-7 cells were incubated with ASO or MSO with LipofectAMINE, and MMP-2 or MMP-9 protein was detected by Western blot, 24 h after transfection. The *left panel* shows the dose-responsive effects of the ASO to MMP-2 (*top panel*) or to MMP-9 (*bottom panel*) to inhibit the intended protein production, but no effect of MSO was found. The *right panel* shows the specificity of an ASO for only its target MMP. Control is in the presence of E2. *B* and *C*, HB-EGF shedding (*B*) or ERK activation (*C*) in response to E2 is prevented by an MMP-2 or MMP-9 ASO but not MSO. HB-EGF shedding was carried out in MCF-7 cells, whereas ERK activation was determined in MCF-7 (*left panel*) or EC (*right panel*). The data are representative of three experiments, except for the *bar graph*, where three experiments are combined. *, $p < 0.05$ for control *versus* E2; +, $p < 0.05$ for E2 *versus* E2 with MMP-2 or MMP-9 ASO.

in the absence) of ERα, E2 stimulated the activation of MMP-2 and MMP-9 (Fig. 6*C*). This was partially dependent upon extracellular calcium, PLC, and PKC signaling. These data strongly support the idea that the classical ERα is required for E2 to activate rapid signaling in breast cancer.

The Role of EGFR in E2 Activation of Multiple Signaling Pathways—Most studies invoking the role of EGFR in GPCR signaling have examined ERK activation. Regarding E2 signal-

incubated with E2 with or without PP2 (soluble Src inhibitor) or with E2 in cells transfected to express a dominant negative Src (pRC-csrc-K298M). By gelatin zymography (see "Experimental Procedures"), active (*act*) MMP-9 and MMP-2 are shown, along with the inactive (*pro*) MMP-2 protein. *M.W.*, molecular weight. *C*, matrix metalloproteinase or ER inhibition prevents E2-induced ERK activation. MCF-7 cells (*left panel*) and ZR-75–1 cells (*right panel*) were incubated with E2 ± ICI182780 or GM6001 (*MMPI*), and ERK activity was determined. EGF was also added as a control. The *bar graph* is three experiments combined. *, $p < 0.05$ for control *versus* E2 or EGF; +, $p < 0.05$ for E2 *versus* E2 with MMPI. *MMPI*, matrix metalloproteinase inhibitor; *MBP*, myelin basic protein.

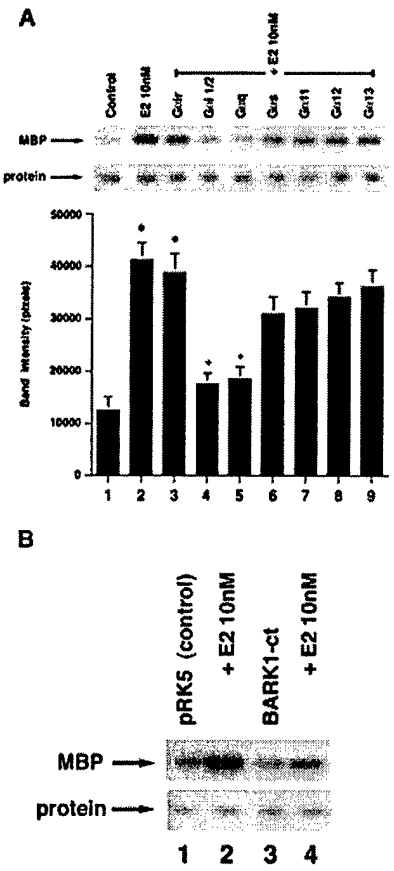


FIG. 5. **Gα subunit protein activation is required for E2-induced ERK activation in MCF-7 cells.** *A*, expression of dominant negative mini-genes for $G\alpha_q$ and Gαi but not $G\alpha_s$, G_{12}, or G_{13} prevents E2-induced ERK. The cells were transfected to express truncated Gα subunits, serving as dominant negatives, then recovered, and exposed to E2 10 nM for 8 min. ERK activity was determined. *Lanes 1* and *2* are nontransfected cells; *lane 3* is E2-stimulated ERK after control plasmid transfection. The *bar graph* represents three experiments combined. *, $p < 0.05$ for control *versus* E2, or control *versus* E2-incubated, $G\alpha_{ir}$-transfected cells. +, $p < 0.05$ for E2-incubated, $G\alpha_{ir}$-transfected cells *versus* E2 in $G\alpha_i$ or $G\alpha_q$ mini-gene expressing cells. *B*, Gβγ contributes to E2-induced ERK activation. The cells were transiently transfected to express a dominant negative, C-terminally truncated β-adrenergic receptor kinase (BARK1-CT), the cells were recovered for 24 h, and then ERK activation by E2 was determined. A representative experiment, repeated twice, is shown.

ing, ERK and cAMP generation are the two pathways that have been identified to require EGFR activation (24, 25), but this has only been established in breast cancer cells. To further define the role of EGFR in E2-induced signaling from membrane ER, we examined breast cancer cells and EC, cells that express endogenous ER (9, 20). In MCF-7 cells, we found that E2 activated protein kinase B (AKT) (Fig. 7*A*). This was substantially prevented by the soluble inhibitor of MMP activity and by tyrphostin AG1478, implicating the EGFR. These two compounds had no effects by themselves (data not shown). In EC, we previously showed that E2 activates the p38β member of the MAP kinase family, and this was essential for E2 to act as a cell survival factor during hypoxia, to preserve EC morphology after metabolic insult, and to stimulate EC migration and primitive capillary formation (33). Here, we show that MMP inhibition or EGFR tyrosine kinase inhibition significantly prevents E2 signaling to the activation of p38β (Fig. 7*B*). Thus, additional signal transduction pathways are rapidly triggered by E2 in several cell types, and these pathways require EGFR transactivation via the linked events we show here.

E Domain Activation of Signaling—What structural aspect of the membrane ER is necessary for activation of the signal cascade that results in EGFR activation and ERK up-regulation? This is an important issue, and assuming that the membrane and nuclear proteins are the same (6), there is no typical catalytic or kinase domain sequence present in ERα or ERβ. It has recently been shown in CHO cells lacking endogenous ER that targeting of the E domain of ERα to the plasma membrane is sufficient to allow strong activation of ERK by E2 (5). This same, localized construct rescues HeLa cells from apoptotic cell death in response to etoposide (17), and in both situations, targeting of the E domain to the nucleus had no effect. We therefore asked whether targeting the E domain of ERα to the plasma membrane was sufficient to activate the signal pathway that we define here. This was accomplished in the HCC-1569 breast cancer cells that do not express ER. Targeting the E domain to the plasma membrane resulted in MMP activation (Fig. 8*A*) and EGFR activation (Fig. 8*B*), leading to ERK up-regulation (5). In the absence of the expressed E domain, E2 was unable to stimulate any of these events. Targeting the E domain to the nucleus also did not result in activation of this pathway. Therefore, the E domain appears to be sufficient for the complex interactions at the plasma membrane that allow assembly and activation of the signalsome in response to E2.

DISCUSSION

The ability of E2 to signal from membrane ER is increasingly appreciated as being important to the effects of this sex steroid. E2 triggers calcium increases in seconds and rapidly activates PKC and adenylate cyclase. Downstream activation of several kinases then leads to cell biological effects in a variety of cell types (44). Ischemia reperfusion injury of muscle in rats is limited by E2, and this occurs through the physical association of ER with phosphatidylinositol 3-kinase, the subsequent up-regulation of kinase activity, and the generation of NO (13). E2 acts as a survival factor for neurons (27), breast cancer (16, 45), and osteoblasts (17) while suppressing osteoclast differentiation (14). The sex steroid also acts as a survival and angiogenesis-promoting factor for EC (33). These effects are related to the modulation of ERK, JNK, and p38 MAP kinases, regulated through membrane ER. Recently, it was shown that the administration of antibodies to ERα in nude mice blocked the growth of human breast cancer xenografts. This probably resulted from the antibodies inhibiting membrane ER signaling to ERK and phosphatidylinositol 3-kinase (46). Therefore, it is important to understand how E2 acts through membrane ER to trigger signal transduction.

Now established for a variety of GPCRs, membrane ER localize substantially to caveolae (3–5). Here, they can physically complex with or activate signal molecules, including G proteins, receptor tyrosine kinases (insulin-like growth factor-1 receptor and EGFR), nonreceptor tyrosine kinases (Src family), and a variety of adapter and threonine/serine kinase proteins. This probably occurs on a scaffold platform provided by caveolin-1 (47, 48) and in part related to tyrosine 14 phosphorylation of this protein (49). Interestingly, this tyrosine is the principal substrate for Src kinase action (49), and the ability of E2 to activate Src at the membrane (8, 17) may therefore contribute to assembling the mature signalsome upon ER ligation by E2. Caveolin-1 can bind to and promote the assemblage of G proteins, Src, Grb7, Raf, Ras, MEK, and the EGFR at the plasma membrane (48). Caveolin-1 also facilitates ER translocation to the plasma membrane and localization within the caveolae microdomain (5). In this way, localizing ER and the signaling molecules to a confined area could augment the ability of E2/ER to transactivate EGFR, resulting in the stimulation of

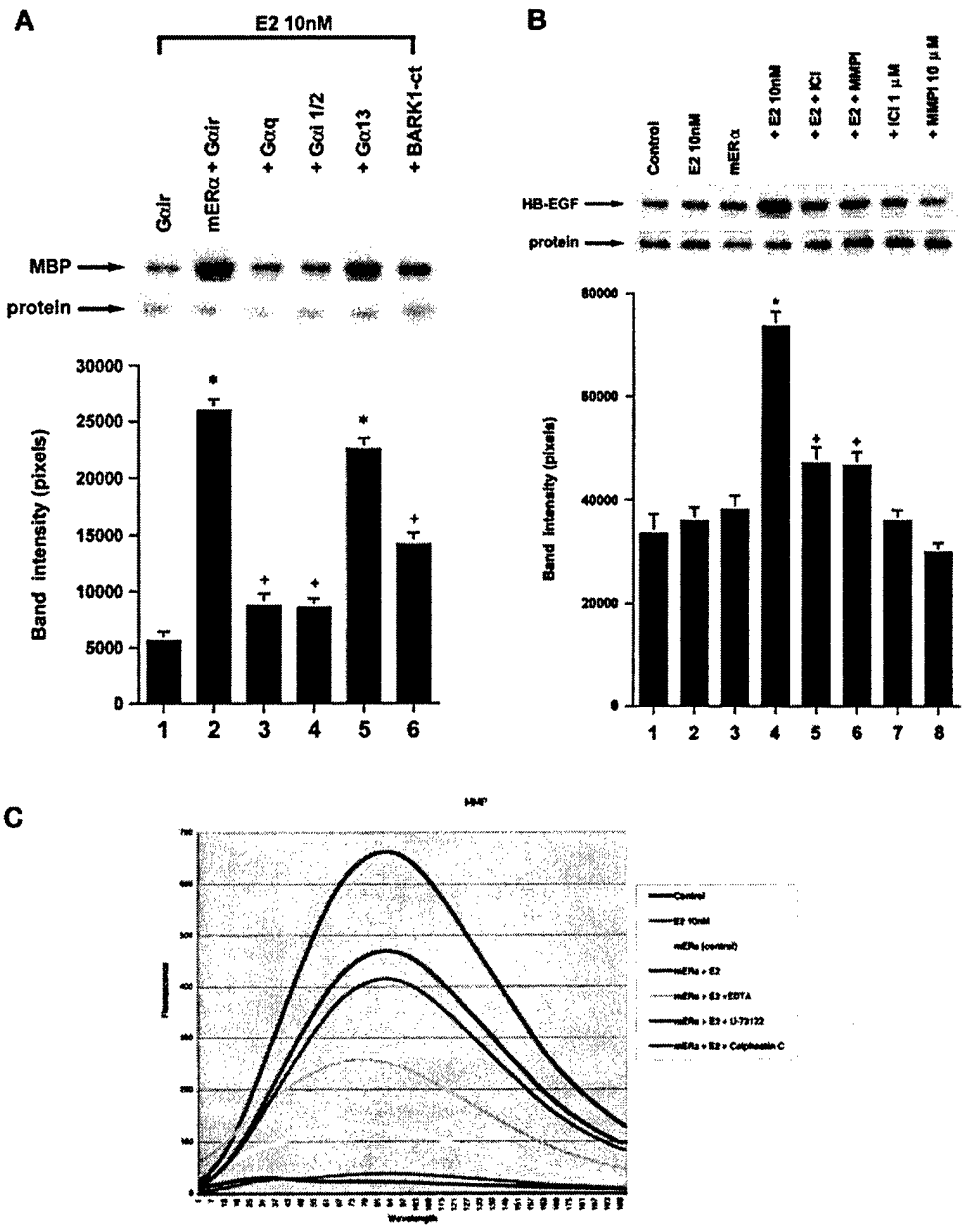


FIG. 6. **ER is necessary for E2-induced proximal signaling.** *A*, specific G protein subunits are required for ER-induced ERK. HCC-1569 cells were co-transfected to express ERα or pcDNA3 and truncated Gα subunits or the C-terminal truncated β-adrenergic receptor kinase (BARK1-CT). The cells were recovered and then incubated with 10nME2 for 10 min, and ERK activity was determined. The *bar graph* represents two experiments combined. *B*, HB-EGF secretion in response to E2. ERα or pcDNA3 was expressed, and the cells were incubated with E2 with or without MMPI or ICI182780 for 3 min. Electrophoresed proteins were then subjected to Western blot. The *bar graph* is three experiments combined. *C*, MMPI activation by E2 requires ER. ERα-transfected HCC-1569 cells were incubated with E2 for 2 min, and the cell lysate was used to determine MMP activity by spectroflurometry. EDTA is a calcium chelator, U-73122 is a PLC inhibitor, and calphostin C is a PKC inhibitor.

ERK activity (this work and Ref. 25). However, upon GPCR ligation, caveolin probably dissociates from binding to the EGFR, leading to the activation of this receptor tyrosine kinase (50).

Although some details of the mechanisms of EGFR transactivation by ER (or any GPCR) are known, there are several aspects that are not clear. We found that the ability of membrane ER to activate $G\alpha_q$ and $G\alpha_i$, but not the α subunits of G_s, G_{12}, and G_{13}, was important to the subsequent (but still rapid) signaling events upstream of EGFR activation (Fig. 9). $G\beta\gamma$ inhibition also prevented the full transactivation of EGFR and ERK up-regulation in response to E2. This underscores the ideas that E2 activates several G proteins (6) and that there are specific functions for each but with some degree of redundancy. The partial redundancy we demonstrate may be related to the requirement that full signaling by E2 requires multiple G protein activations. Supporting this idea, we found that co-expressing dominant negative mini-genes for $G\alpha_q$ and $G\alpha_i$ (but not co-expression with GαS) added to the inhibition of E2/ER signaling to MMP activation and EGFR activation, compared with the inhibition of single Gα subunits.[2] This may be related to the necessity for complete activation of Src and Src-induced signaling to MMP activation (see below).

[2] M. Razandi, A. Pedram, and E. R. Levin, unpublished observations.

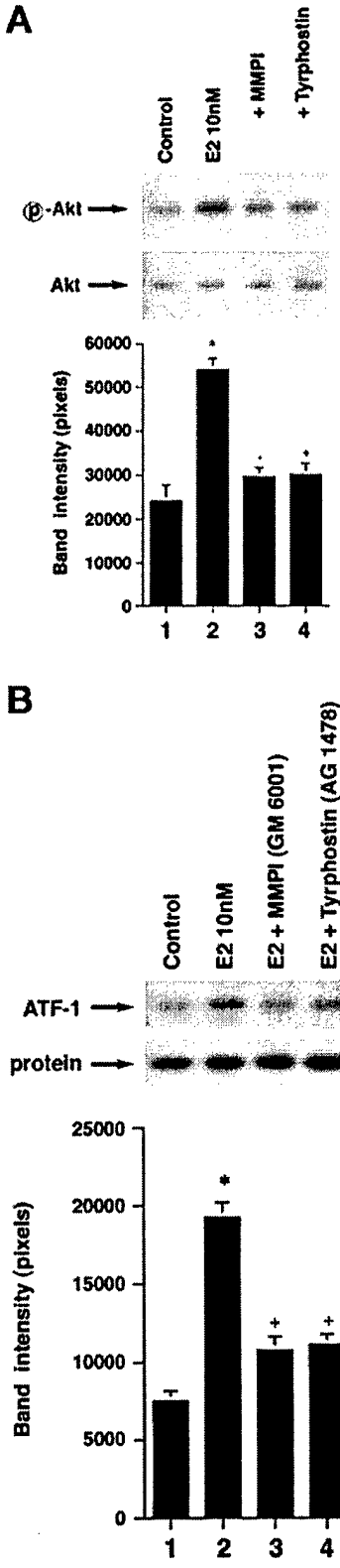


FIG. 7. **Additional signaling pathways that depend upon ER to EGFR cross-talk.** *A*, E2-induces AKT activation in MCF-7 cells, dependent upon MMP activation and EGFR tyrosine kinase function. MCF-7 cells were incubated with E2 with or without GM6001 or tyrphostin AG1478 for 10 min, and AKT phosphorylation at serine 473 was determined. *B*, p38β activation in endothelial cells by E2 is significantly prevented by inhibition of MMP activity or the EGFR tyrosine kinase. EC were incubated with 10 nM E2 for 15 min, and the p38 immunoprecipitated from the cell lysate(s) was used for *in vitro* kinase activity assays, with ATF-1 as substrate. The *bar graphs* are from three experiments combined.

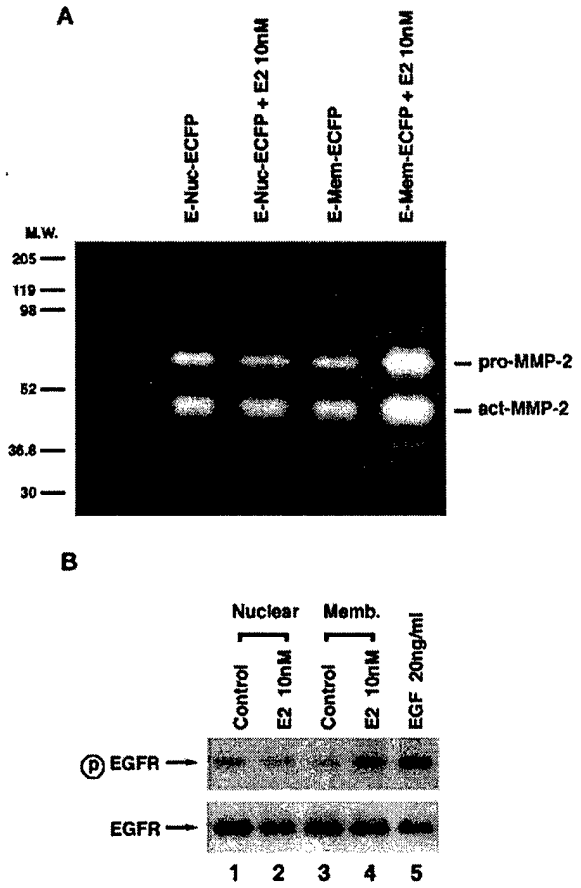


FIG. 8. **Structure/function relationship between ER and signaling.** *A*, targeting the E domain of ERα to the membrane of breast cancer cells augments MMP activity. HCC-1569 cells were transiently transfected to express the E domain targeted to the nucleus (*lanes 1* and *2*) or to the plasma membrane (*lanes 3* and *4*), followed by 2 min of treatment with E2 and determination of MMP activity. A representative study is shown, repeated once. *B*, expressing the E domain in the membrane leads to the transactivation of EGFR by E2. The transfected cells were assayed for EGFR phosphorylation by Western Blot, using an antibody against tyrosine 1138 (*lane 3 versus lane 4*). EGF-induced transactivation of its receptor serves as a positive control. The study was repeated twice.

We also found that MMP-2 and MMP-9 were necessary for E2 to stimulate the secretion of HB-EGF and the transactivation of EGFR. First, E2 activated these two enzymes, as determined by gelatin zymography and substrate cleavage studies. However, E2 did not up-regulate MMP-3 and MMP-13 activity, thus showing the specificity of our results. Second, E2 induced the release of HB-EGF into the cell culture medium after only 3 min of incubation, and this was substantially prevented by the specific antisense (but not missense) constructs for MMP-2 and MMP-9, with the effects being additive. Shedding of HB-EGF is a complicated process, and involvement of Ras-Raf-Mek (51), Rac (52), or PKCδ and the metalloprotease-disintegrin, MDC9 (37), has been proposed. In some cellular contexts, unknown metalloproteinase(s) mediates this shedding (53). Recently, TACE/ADAM17 has been shown to cleave expressed HB-EGF at 24 h in fibroblasts (54). However, our results suggest that MMP-2 and MMP-9 are sufficient.

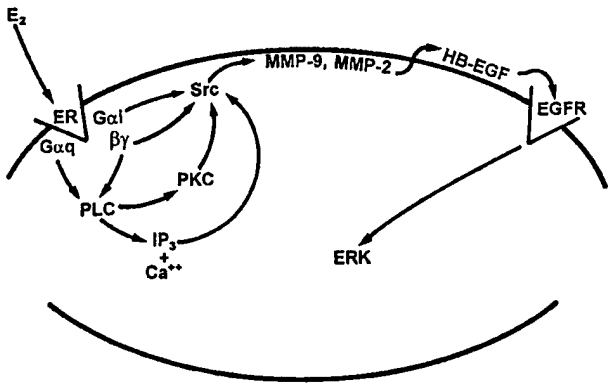


FIG. 9. **E2-induced proximal signaling to the transactivation of EGFR, leading to ERK activation.**

The specific signal from GPCRs that leads to MMP activation is not well understood. In this regard, we report the novel finding that Src is necessary for E2 to activate MMP-2 and MMP-9 and subsequent HB-EGF shedding. A previous study implicated Src as upstream from HB-EGF, but its role was undetermined (29). The precise mechanisms by which Src accomplishes MMP secretion and activation is unknown but is under investigation. It should be appreciated that this kinase is also *downstream* of EGFR, either through Src binding this receptor or through potential cross-talk of EGFR to G protein-coupled receptors, leading to Src activation (55, 56).

Our novel identification of MMP-2 and MMP-9 secretion and activation as being involved in estrogen signaling underlies the overall contribution of these MMPs to breast cancer biology. MMP-2 and MMP-9 have been implicated in the aggressive behavior of breast cancer (57, 58). The ability of breast cancer cells to migrate or invade/metastasize is importantly dependent on the degradation of cell matrix by MMPs. However, these proteases also play additional important roles to mediate cell survival, differentiation, and angiogenesis (reviewed in Ref. 59). Recently, MMP-2 production in response to E2 was found to be dependent on ERK signaling to the up-regulated activity of the AP-2 transcription factor in mesangial cells (60). In our model, MMP activation is necessary for E2 to stimulate HB-EGF secretion into the culture media, and HB-EGF but not TGFα transactivates the EGFR to signal to ERK.

The ability of EGFR to underlie E2/ER-induced ERK may represent only a single example of the wider signaling interactions of these two growth modulatory systems. We therefore asked whether other important signaling pathways that originate from membrane ER are also dependent on EGFR. We report here that E2 activates protein kinase B in breast cancer cells and p38β MAP kinase in EC and that both pathways are also dependent upon transactivation of the EGFR. Utilization of EGFR to activate ERK is relatively common for a variety of GPCRs (38, 61); however, GPCRs can also activate ERK by pathways apart from EGFR (62). In this respect, we previously showed that E2 stimulates $G\alpha_s$ and cAMP, as well as ERK in CHO cells that are transiently transfected to express ER but that lack endogenous EGFR (6). Other EGF receptor family members might facilitate GPCR signaling to distinct pathways and thereby contribute to the specificity of signaling. For instance, the ability of muscarinic receptors to activate AKT is dependent upon the transactivation of the ErbB3 member of the EGFR family (63). In other situations, platelet-derived growth factor or insulin-like growth factor-1 receptors may be necessary for GPCR effects (64–66). Thus, the tyrosine kinase receptor milieu in a particular cell may specifically control the panoply of signaling typically enacted by a GPCR ligand.

These interactions extend to cross-talk in both directions, including from the growth factor receptor tyrosine kinase to ER (67). Insulin-like growth factor-1 and EGF can signal to transcription via ER, independently of E2 (68, 69). This occurs through growth factor receptor-induced phosphorylation of the nuclear sex steroid receptor (70) or co-accessory proteins (71), and the induction of several kinase cascades is important in this regard (70, 72). These complex interactions are important in that they may contribute to the ability of breast cancer cells to proliferate or survive via ER, even when circulating levels of E2 are low, as in the post-menopausal woman.

An additional important issue is whether ER is required for E2 to activate signaling pathways from the membrane. It has previously been shown that E2 can transactivate the EGFR and signal in breast cancer cells that do not express ER (24). This purportedly occurs through a nondefined interaction with the orphan GPCR, GPR 30, and can nonspecifically be activated by estrogen receptor antagonists (ICI182780) and relatively inactive steroisomers (17-α-E2). We report here that in the absence of ER, E2 can not activate ERK in HCC-1569 cells that lack this receptor. Expressing ER (or the E domain targeted to the cell membrane) allows E2 to signal through specific G proteins, MMP activation, and HB-EGF secretion that activates EGFR. Although some cells have been reported to respond rapidly to E2 in a nontraditional ER-related or ER-independent fashion (10, 73), the mechanisms underlying these reports remain unknown. Furthermore, the majority of studies indicate the requirement of ER for E2 action (5, 6, 8, 20, 27, 28), and these studies identify typical receptor pharmacology for the nongenomic actions of this sex steroid (reviewed in Ref. 43).

What part of ER is necessary for signal transduction at the membrane? Tyrosine 537 in the AF-2 portion of the E domain is essential for the interaction of ER with Src and functional up-regulation of ERK in MCF-7 cells (8). Recently, an interaction between the AF-1 domain of ERα and the phosphotyrosine binding and SH2 domains of the adapter protein Shc was postulated to mediate ERK activation in MCF-7 cells (20). However, Razandi *et al.* (5) recently showed that targeting the E domain of ERα to the plasma membrane of CHO cells is sufficient for robust ERK activation by E2, and Kousteni *et al.* (17) showed that this was sufficient to rescue HeLa cells from apoptosis. Here, we show that targeting the E domain to the membrane (and not to the nucleus) of HCC-1569 cells results in MMP-2 activation, EGFR transactivation, and ERK up-regulation. Thus, it is not clear what the significance of the AF-1 region of ER might be for signaling from the membrane. We propose that elements in the E domain, such as AF-2, allows for the complex interactions with G proteins, caveolin, Src, and other signaling molecules.

In summary, E2 activation of ERK is dependent on several Gα and G$\beta\gamma$ subunits of small GTP-binding proteins. Src-dependent stimulation of MMP-2 and MMP-9 activity in response to E2/ER releases HB-EGF, leading to EGFR transactivation, and signaling to MAP kinase. The E domain of ERα appears to be sufficient to activate these mechanisms. The assemblage of signal transduction complexes probably platformed on caveolin or growth factor receptor tyrosine kinase proteins (EGFR and insulin-like growth factor receptor) accounts for much of the ability of E2 to signal through membrane-localized ER to different pathways. This mechanism is increasingly appreciated to play important roles in the cellular biology of E2 actions, and manipulation of these

pathways could therapeutically modulate the effects of this sex steroid.

REFERENCES

1. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
2. Falkenstein, E., and Wehling, M. (2000) *Eur. J. Clin. Invest.* **3,** (suppl.) 51–54
3. Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999) *Biochem. Biophys. Res. Commun.* **263,** 257–262
4. Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., Mendelsohn, M. E., Anderson, R. G. W., and Shaul, P. W. (2000) *Circ. Res.* **87,** e44–e52
5. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) *Mol. Endocrinol.* **16,** 100–115
6. Razandi M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13,** 307–319
7. Wyckoff, M. H., Chambliss, K. L., Mineo, C., Yuhanna, I. S., Mendelsohn, M. E., Mumby, S. M., and Shaul, P. W. (2001) *J. Biol. Chem.* **276,** 27071–27076
8. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Vitorria Barone, M., Ametrano, D., Zannini, M. S., Abbondanza, C., Bontempo, P., and Auricchio, F. (2000) *EMBO J.* **19,** 5406–5417
9. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) *EMBO J.* **15,** 1292–1300
10. Singh, M., Setalo, G., Jr., Guan, X., Warren, M., and Toran-Allerand, C. D. (1999) *J. Neurosci.* **19,** 1179–1188
11. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997) *J. Biol. Chem.* **272,** 11902–11907
12. Ansonoff, M. A., and Etgen, A. M. (1998) *Endocrinology* **139,** 3050–3056
13. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) *Nature* **407,** 538–541
14. Shevde, N. K., Bendixen, A. C., Dienger, K. M., and Pike, J. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7829–7834
15. Morey, A. K., Pedram, A., Razandi, M., Prins, B. A., Hu, R.-M., Biesiada, E., and Levin, E. R. (1997) *Endocrinology* **138,** 3330–3339
16. Razandi, M., Pedram, A., and Levin, E. R. (2000) *Mol. Endocrinol.* **14,** 1434–1447
17. Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2001) *Cell* **104,** 719–730
18. Pappas, T. C., Gametchu, B., Yannariello-Brown, J., Collins, T. J., and Watson, C. S. (1995) *FASEB J.* **9,** 404–410
19. Norfleet, A. M., Thomas, M. L., Gametchu, B., and Watson, C. S. (1999) *Endocrinology* **140,** 3805–3814
20. Russell, K. S., Haynes, M. P., Sinha, D., Clerisme, E., and Bender, J. R. (2000) *(2000) Proc. Natl. Acad. Sci. U. S. A.* **97,** 5930–5935
21. Song, R. X., McPherson, R. A., Adam, L., Bao, Y., Shupnik, M., Kumar, R., and Santen, R. J. (2002) *Mol. Endocrinol.* **16,** 116–127
22. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8517–8522
23. Lieberherr, M., Grosse, B., Kachkache, M., and Balsan, S. (1993) *J. Bone Min. Res.* **8,** 1365–1376
24. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., and Bland, K. I. (2002) *Mol. Endocrinol.* **16,** 70–84
25. Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R. (2000) *Mol. Endocrinol.* **14,** 1649–1660
26. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 19443–19450
27. Singer, C. A., Figueroa-Masot, X. A., Batchelor, R. H., and Dorsa, D. M. (1999) *J. Neurosci.* **19,** 2455–2463
28. Zhu, Y., Bian, Z., Lu, P., Karas, R. H., Bao, L., Cox, D., Hodgin, J., Shaul, P. W., Thoren, P., Smithies, O., Gustafsson, J. A., and Mendelsohn, M. E. (2002) *Science* **295,** 505–508
29. Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **22,** 23155–23160
30. Pedram, A., Razandi, M., and Levin, E. R. (1998) *J. Biol. Chem.* **273,** 26722–26728
31. Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O., and Korach, K. S. (1995) *Mol. Endocrinol.* **9,** 1441–1454
32. Redemann, A., Holzmann, B., von Ruden, T., Wagner, E. F., Schlessinger, J., and Ullrich, A. (1992) *Mol. Cell. Biol.* **12,** 491–498
33. Razandi, M., Pedram, A., and Levin, E. R. (2000) *J. Biol. Chem.* **275,** 38540–38546
34. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269,** 6193–6197
35. Gilchrist, A., Li, A., and Hamm, H. E. (2002) *Methods Enzymol.* **344,** 58–69
36. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402,** 884–888
37. Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S., and Mekada, E. (1998) *EMBO J.* **17,** 7260–7272
38. Duffy, M. J., Maguire, T. M, Hill, A., McDermott, E., and O'Higgins, N. (2000) *Breast Cancer Res.* **2,** 252–257
39. Hua, J., and Muschel, R. J. (1996) *Cancer Res.* **56,** 5279–5284
40. Perret, S., Dockery, P., and Harvey, B. J. (2001) *Mol. Cell. Endocrinol.* **176,** 77–84
41. Rubio-Gayosso, I., Sierra-Ramirez, A., Garcia-Vazquez, A., Martinez-Martinez, A., Munoz-Garcia, O., Morato, T., and Ceballos-Reyes, G. (2000) *J. Cardiovasc. Pharmacol.* **36,** 196–202
42. Kelly, M. J, Wagner, E. J. (1999) *Trends Endocrinol. Metab.* **10,** 369–374
43. Sylvia, V. L., Walton, J., Lopez, D., Dean, D. D., Boyan, B. D., and Schwartz, Z. (2001) *J. Cell. Biochem.* **81,** 413–429
44. Levin, E. R. (2001) *J. Applied Physiol.* **91,** 1860–1867
45. Teixeira, C., Reed, J. C., and Pratt, M. A. (1995) *Cancer Res.* **55,** 3902–3907
46. Marquez, D. C., and Pietras, R. J. (2001) *Oncogene* **20,** 5420–5430
47. Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997) *J. Biol. Chem.* **272,** 7211–7222
48. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273,** 5419–5422
49. Lee, H., Volonte, D., Galbiati, F., Iyengar, P., Lublin, D. M., Bregman, D. B., Wilson, M. T., Campos-Gonzalez, R., Bouzahzah, B., Pestell, R. G., Scherer, P. E., and Lisanti, M. P. (2000) *Mol. Endocrinol.* **14,** 1750–1775
50. Ushio-Fukaki, M., Hilenski, L., Santanam, N., Becker, P. L., Ma, Y., Griendling, K., and Alexander, R. W. (2001) *J. Biol. Chem.* **276,** 48269–48275
51. Gechtman, Z., Alonso, J. L., Raab, G., Ingber, D. E., and Klagsbrun, M. (1999) *J. Biol. Chem.* **274,** 18828–18835
52. Umata, T., Hirata, M., Takahashi, T., Ryu, F., Shida, S., Takahashi, Y., Tsuneoka, M., Miura, Y., Masuda, M., Horiguchi, Y., and Medada, E. (2001) *J. Biol. Chem.* **276,** 30475–30482
53. Dong, J., Opresko, L. K., Dempsey, P. J., Lauffenburger, D. A., Coffey, R. J., and Wiley, H. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6235–6240
54. Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschon, J. J., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A., and Lee, D. C. (2002) *J. Biol. Chem.* **277,** 12838–12845
55. Seta, K., Nanamori, M., Modrall, J. G., Neubig, R. R., and Sadoshima, J. (2002) *J. Biol. Chem.* **277,** 9268–9277
56. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) *Curr. Opin. Cell Biol.* **11,** 177–183
57. Westermarck, J., and Kahari, V.-M. (1999) *FASEB J.* **13,** 781–792
58. Ray, J. M., and Stetler-Stevenson, W. G. (1994) *Eur. Respir. J.* **7,** 2062–2072
59. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) *Science* **295,** 2387–2392
60. Guccione, M., Silberger, S., Lei, J., and Nuegarten, J. (2002) *Am. J. Physiol.* **282,** F164–F169
61. Hackel, P. O., Zwick, E., Prenzel, N., and Ullrich, A. (1999) *Curr. Opin. Cell Biol.* **11,** 184–189
62. Andreev, J., Galisteo, M. L., Kranenburg, O., Logan, S. K., Chiu, E. S., Okigaki, M., Cary, L. A., Moolenaar, W. H., and Schlessinger, J. (2001) *J. Biol. Chem.* **276,** 20130–20135
63. Tang, X., Batty, I. H., and Downes, C. P. (2002) *J. Biol. Chem.* **277,** 338–344
64. Schmidt, M., Frings, M., Mono, M. L., Guo, Y., Weernink, P. A., Evellin, S., Han, L., and Jakobs, K. H. (2000) *J. Biol. Chem.* **275,** 32603–32610
65. Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000) *J. Biol. Chem.* **275,** 18447–18453
66. Sumitomo, M., Milowsky, M. I., Shen, R., Navarro, D., Dai, J., Asano, T., Hayakawa, M., and Nanus, D. M. (2001) *Cancer Res.* **61,** 3294–3298
67. Yee, D., and Lee, A. V. (2000) *J. Mammary Gland Biol. Neoplasia* **5,** 107–115
68. Klotz, D. M., Curtis Hewitt, S., Ciana, P., Raviscioni, M., Lindzey, J. K., Foley, J., Maggi, A., DiAugustine, R. P., and Korach, K. S. (2002) *J. Biol. Chem.* **277,** 8531–8537
69. Curtis, S. W., Washburn, T., Sewall, C., DiAugustine, R., Lindzey, J., Couse, J. F., and Korach, K. S. (1996) *Proc. Natl. Acad. Sci.* **93,** 12626–12630
70. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491–1494
71. Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. (2001) *J. Biol. Chem.* **276,** 22177–22182
72. Martin, M. B., Franke, T. F., Stoica, G. E., Chambon, P., Katzenellenbogen, B. S., Stoica, B. A., McLemore, M. S., Olivo, S. E., and Stoica, A. (2000) *Endocrinology* **141,** 4503–4511
73. Guo, Z., Krucken, J., Benten, W. P., and Wunderlich, F. (2002) *J. Biol. Chem.* **277,** 7044–7050

0888-8809/03/$15.00/0
Printed in U.S.A.

APPENDIX 3

Molecular Endocrinology 17(3):309–317

doi: 10.1210/me.2002-0368

MINIREVIEW

Bidirectional Signaling between the Estrogen Receptor and the Epidermal Growth Factor Receptor

ELLIS R. LEVIN

Division of Endocrinology, Long Beach Veterans Affairs Medical Center, Long Beach, California 90822; and Departments of Medicine and Pharmacology, University of California, Irvine, California 92717

Interactions between the estrogen receptor (ER) and the epidermal growth factor receptor (EGFR) contribute to the biological effects of these binding protein families. EGFR stimulates DNA synthesis and gene transcription in the uterus, related in part to estrogen-independent activation of the nuclear ER. This results from signal transduction enacted by the plasma membrane tyrosine kinase growth factor receptor, leading to 1) phosphorylation and activation of the nuclear ER, and 2) phosphorylation of coregulator proteins. More recently, it has been shown that a pool of ERα resides in or associates with the plasma membrane as a cytoplasmic protein. These ERs utilize the membrane EGFR to rapidly signal through various kinase cascades that influence both transcriptional and nontranscriptional actions of estrogen in breast cancer cells. This is congruent with a general theme of receptor signaling, where membrane G protein-coupled receptors activate tyrosine kinase growth factor receptors (EGFR, IGF-I receptor) that subsequently signal to MAPKs and other pathways. Overall, the bidirectional cross-talk between EGFR and cellular pools of ER contributes to reproductive organ physiology and pathophysiology. (*Molecular Endocrinology* 17: 309–317, 2003)

MOST GROWTH FACTORS activate cell proliferation, differentiation, or survival programs through binding their attendant tyrosine kinase receptors, expressed in the plasma membrane (1–4). As a result, the receptors undergo dimerization and conformational changes that result in transphosphorylation at discrete tyrosine residues. This provides binding sites for signaling or linker/adapter molecules that contain Src-homology 2 domains, and the recruitment of additional signal molecules (5, 6). Such proteins include nonreceptor tyrosine kinases such as Src family members, or Grb and Sos family proteins. Signal cascades are then triggered, dependant upon the translocation, membrane association, and activation of tyrosine, serine/threonine, and lipid kinases, including ras, raf, protein kinase C, and phosphatidylinositol 3-kinase (PI3K). These kinases phosphorylate substrate proteins in the cytoplasm, altering target protein function. As an example, stimulation of PI3K results in AKT activation, which then phosphorylates a variety of proapoptotic proteins, including BAD, glycogen synthase kinase-3β, or Forkhead transcription factors (7, 8). This posttranslational modification sequesters/inactivates these proteins in cytoplasm, leading to cell survival.

Kinases also translocate to the nucleus, where they phosphorylate/activate and transcribe transcription factors that induce a variety of immediate early and late-arising genes. This important event underlies many of the biological effects of growth factor signaling. In fact, when nuclear localization of the ERK member of the MAPK family is prevented, cell proliferation often ceases (9). Important nuclear targets of ERK that are relevant to cell division include the transactivation of the cyclin D1 gene and the protooncogenes c-*fos* and c-*myc* (10–13). Therefore, the ability to signal from the membrane to both cytoplasmic and nuclear events is an essential feature of growth factor receptor function.

Steroid hormones have traditionally been conceived to act through the ligation of nuclear receptors (14). For estrogen, binding to estrogen receptor (ER)α or ERβ results in an active complex in the nucleus that binds DNA directly at estrogen response elements within the promoters of target genes. Alternatively, estradiol (E_2)/ER promotes transcription factor binding to DNA (15). Liganded ER forms complexes with coregulator proteins (16), and constituents of the basal

Abbreviations: E_2, Estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; GPCR, G protein-coupled receptor; HB-EGF, heparin binding-EGF; IGF-IR, IGF-I receptor; KO, knockout; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription.

transcription machinery complex, leading to the modulation of RNA polymerase II activity, histone-induced chromatin unwinding, and transcription. However, it has become increasingly clear that estrogen (and other steroid hormones) also rapidly activates signaling in seconds to minutes, and this cannot be explained by any known function of nuclear receptors (17). Furthermore, ERs that lack a nuclear localization sequence (18) or truncated ERs that are targeted to the plasma membrane are fully capable of activating kinases and subsequent cell proliferation or survival (19, 20). A small pool of endogenous ERs that localize to the plasma membrane in various target cells can act similarly to classic growth factor receptors imbedded in the membrane. These ERs have been localized to caveolae raft domains isolated from the plasma membrane of target cells such as endothelial cells (19, 21). It is still unclear, however, whether these sex steroid receptors are integral membrane proteins and/or tether as cytoplasmic proteins to the cytoplasmic face of caveolae through binding to caveolin-1.

An important principle in the signaling field is that growth factor receptors cross-talk to each other. This includes heterodimerization between receptor family members, exemplified by the four members of the epidermal growth factor (EGF) receptor (EGFR) family (22). Additionally, signaling from one receptor activates cytoplasmic nonreceptor kinases (*e.g.* -Src) that positively or negatively modulate the activity of adjacent receptors (23). In this respect, EGFRs expressed on a population of cells may spread signal transduction enacted by a variety of unrelated growth factor receptors on adjacent cells (24).

Emerging data suggest cross-talk may exist between plasma membrane steroid receptors. Progesterone can stimulate ERK signaling via the utilization of ER (25), and estrogen or androgen can promiscuously stimulate signaling to ERK (and cell survival) through either sex steroid receptor (20). Furthermore, both membrane growth factor and steroid receptors interweave their actions with those of nuclear steroid receptors, thereby impacting cell biology. An example is that nuclear receptors transcribe genes, the protein products of which are acutely altered in function by phosphorylation, resulting from membrane receptor signaling.

In this overview, I will describe the current state of cross-talk between ERs and EGFRs. Work in this area has established a requirement of nuclear ER for some EGFR [and perhaps IGF-I receptor (IGF-IR)] actions. Recent findings suggest the important role of EGFR (or similar receptors) for estrogen signaling from the membrane in breast cancer. Bidirectional signaling between these essential cellular factors augments the actions of the individual steroid and growth protein.

SIGNALING FROM EGFRs TO NUCLEAR ERs

EGF binds to one or more members of the EGFR family that enact signaling cascades to the nucleus and cytoplasm, resulting in cell biological actions (22, 26). This pathway is indirectly used by E_2. In reproductive organs, E_2 induces the EGFR and stimulates growth and rapid proteolytic activity in the uterus (27). Subsequent investigations established that this sex steroid stimulates the synthesis of EGF in this reproductive organ (28). Up-regulation of EGF probably explains the strong proliferative effect of E_2 on uterine epithelium, an action that is prevented by EGF antibody (29). Increased synthesis of EGF resulting from E_2 action extended the earlier observation that E_2 induces EGF secretion from breast cancer cells (30) and implicates this interaction in the growth of hormonally responsive cancer. In EFGR knockout (KO) mice, the stromal compartment, but not the epithelial response to E_2, is severely limited in both the uterus and vagina (31).

A novel model of ER and EGFR interaction is derived from the observation that EGFR signaling depends upon an ER-mediated function but in an estrogen-independent fashion (32). Studies from Ignar-Trowbridge *et al.* (33) showed that EGF induction of DNA and lipid synthesis in the uterus could be prevented by ICI 164,384, an ER antagonist. More recent studies suggest that the effects of ER antagonists could be mediated through recruiting corepressors, thereby inhibiting growth factor-induced ER transcriptional effects (34). Continued work from the laboratories of DiAugustine and Korach (35) showed that EGF-induced DNA synthesis and transcription were absent in uteri from ERα KO (ERKO) mice. These results clearly show dependency on ER for EGF action in reproductive organs.

How does EGFR utilize ER for biological actions? Insight resulted from the observations that several peptide growth factor receptors signal to the phosphorylation and activation of the nuclear ER (36, 37). This includes EGF and was originally attributed to the ability of growth factor receptor-activated MAPK (ERK) to phosphorylate serine 118 in the A/B domain of the nuclear ERα. Serine 118 phosphorylation results in an increased ER-related transactivation of genes that are up-regulated by EGFR. Work by Ignar-Trowbridge *et al.* (38) showed that EGFR ligation induces the transcriptional up-regulation of an estrogen response element reporter construct, in ER-dependent fashion. This group also demonstrated that EGFR-to-ER cross-talk requires the A/B domain of ERα (39). Subsequent studies implicated several kinases that phosphorylate additional residues within ERα, resulting in the increased transcriptional activity of the nuclear receptor (40–44). Thus, impact of the growth factor receptor-ER interaction depends upon the signaling milieu within a particular cell that differentially phosphorylates numerous residues in the nuclear ER.

Another mechanism through which EGFR-induced signaling modulates ER transcriptional activity is via coregulator protein phosphorylation. As an example, EGF-induced ERK phosphorylates serine 736 of glucocorticoid receptor interacting protein 1. This increases the activity of this nuclear receptor nonspecific coactivator protein (45). EGF-triggered Src and Jnk activation may have a similar function for the cAMP response element-binding protein (46). Other coactivator proteins that are important and specific to ER function could be similarly activated or recruited through signaling-induced posttranslational modifications. Interestingly, growth factor receptors signal to cyclin D1 production, as part of promoting G_1/S phase cell cycling (47). Cyclin D1 activates ER transcriptional function (48) and interacts with the coactivator proteins, steroid receptor coactivator 1 and cAMP response element-binding protein/p300, as an additional mechanism to amplify nuclear ER action (49, 50).

It is conceivable that EGFR signaling also inhibits the activity/function of corepressor proteins on targeted promoters, and that other EGFR family members could also cross-talk to the nuclear ER. In breast cancer and other estrogen target cells, EGFR family members often heterodimerize, and ligands [heparin binding-EGF (HB-EGF), TGFα, or EGF] can be somewhat promiscuous in their binding. Such considerations may be relevant to the interactions of the erb2/Neu oncogene and ER in early breast cancer. In this respect, breast tumor formation in mouse mammary tumor virus-erb2/neu mice is delayed on an ERKO background (51). A summary of mechanisms of EGFR signaling through ER is seen in Fig. 1.

Finally, it has recently been reported that the EGFR translocates to the nucleus, where it can bind to AT-rich DNA sequences and modulate the transcription of the cyclin D1 gene (52). Modulation of this controversial event by EGF occurs 48 h after ligation (53), and any interactions with the nuclear ER would be expected to impact the more chronic effects of the growth factor receptor.

In parallel to the interaction between the EGF system and ER, there is abundant evidence indicating cross-talk between the IGF-I system and ER. IGF-I binding activates its receptor, leading to PI3K/AKT activation, increased ERα synthesis, and augmented ERα transcriptional activity. This probably results from the phosphorylation of several serine residues in the AF-1 region (43) and EGFR accomplishes a similar action. Similarly to EGF, IGF-I activates parameters of

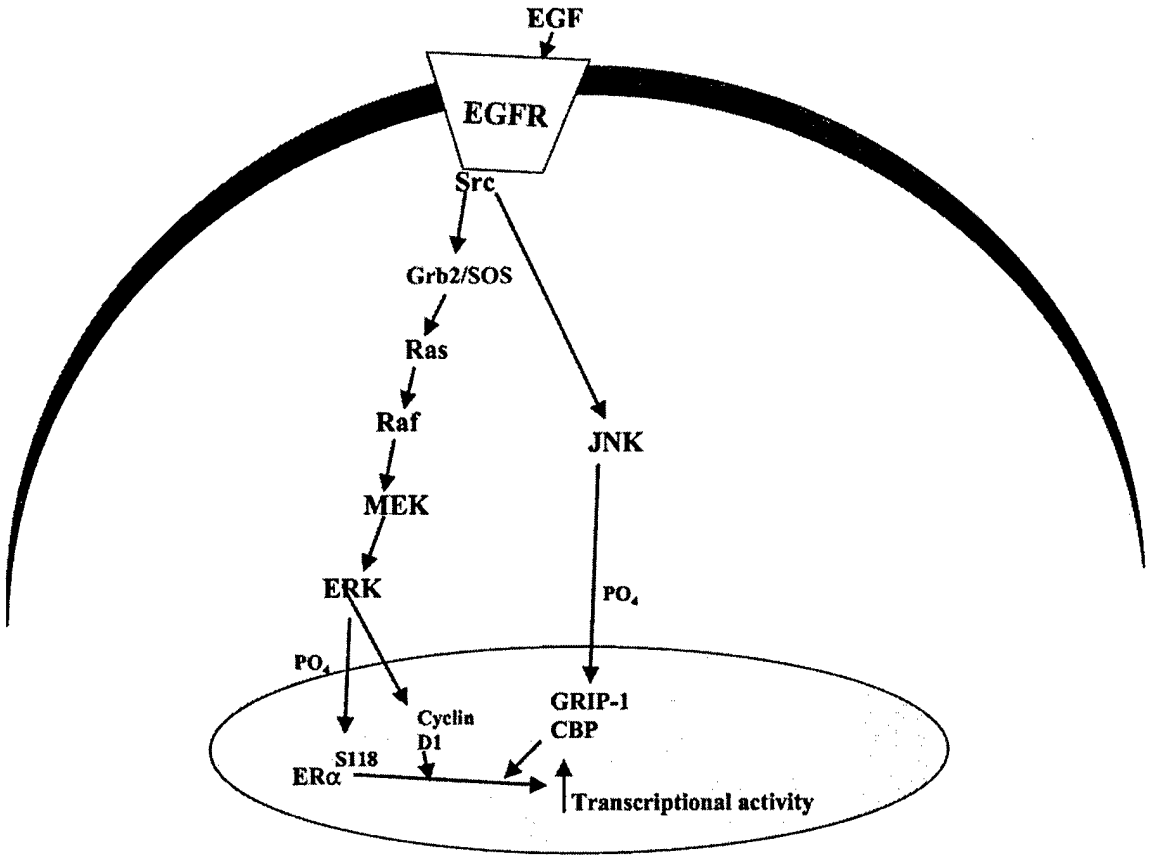


Fig. 1. EGFR Activation of ER or Coregulator Proteins via Signaling through MAPK Cascades
GRIP-1, Glucocorticoid receptor-interacting protein; CBP, cAMP response element-binding protein.

uterine cell proliferation *in vivo*, and this is dependent on ERα (54). Interestingly, in both the uterus and in breast cancer models, IGF-I signaling to ERK and PI3K/AKT is unaffected by ERα loss or antagonism (54, 55). When ERα is reexpressed in breast cancer cells that have lost ER through repetitive culturing, both E_2 and IGF-I resume their growth-inducing function (56). E_2 stimulates many proteins in the IGF-I system, including IRS proteins, IGF-IR, and IGF-binding proteins (57, 58), and ERα binds and phosphorylates the IGF-1R and enhances signaling through the growth factor receptor (59). In breast cancer, IGF-I and E_2 cooperate to promote G_1/S cell cycle progression (60, 61), and in the uterus of the IGF-I KO mouse, E_2-induced growth is absent (62). Thus, there appears to be an important cooperation and cross-talk between these two systems as well.

SIGNALING FROM ER THROUGH EGFR

The realization that E_2 has rapid effects in cells led to the characterization of the many generated signals. E_2 stimulates calcium channel opening and calcium influx or mobilization within seconds of binding receptors expressed in target tissues (63, 64). E_2 rapidly generates cAMP (65), phospholipase C, and inositol phosphate (66, 67). This results from G protein activation, and these early signals are transmitted to the rapid stimulation of protein kinase C, protein kinase A, MAPK, and PI3K (68). Functional and immunohistological identification of endogenous membrane ER (69, 70) led to the characterization of these receptors after expression of the cDNAs for classical ERα and ERβ in Chinese hamster ovary cells (71). These latter studies indicated that membrane ER physically associate with and activate various G protein α-subunits, including Gαs and Gαq. G protein activation explains how ER generates cAMP (Gαs function) or inositol 1,4,5-triphosphate and calcium (Gαq function), as examples. Subsequent work showed that endogenous ERα activates Gαi, leading to the generation of nitric oxide in endothelial cells (72).

An important finding described by Ullrich and colleagues (73) indicates that several G protein-coupled receptors (GPCRs) signal to ERK via the transactivation of the EGFR. Later studies from other laboratories confirmed and extended these observations to many GPCRs and provided additional details underlying this cross-talk. Identification of the membrane ER as a receptor capable of activating G proteins (71, 72) invoked the possibility that this receptor signaled through cross-talk/activation of the membrane EGFR. Filardo *et al.* (74, 75) showed that estrogen rapidly acts in breast cancer cells to stimulate the transactivation of EGFR, leading to cAMP and ERK up-regulation. This occurs through a linked path, first described for other GPCRs by Ullrich and colleagues (76). E_2 induces mainly unknown proximal signaling to cause the activation of undefined matrix metalloproteinases (MMPs). Increased MMP function leads to the liberation of HB-EGF, which then binds and activates the EGFR. However, Filardo *et al.* (74) reported that 17β-E_2, 17α-E_2, or the ER antagonist, ICI 182780, were equivalent in activating EGFR and ERK. EGFR transactivation was proposed to occur independently of any ER and resulted from an undetermined effect of E_2 to activate the orphan GPCR, GPR 30 (77). More recent studies from Razandi *et al.* (78) demonstrated that E_2 requires an ER to signal to EGFR in breast cancer and is consistent with most studies that show an ER is necessary for rapid signaling by E_2 at the membrane (19, 42, 66, 79–81). Razandi *et al.* (78) also found that E_2/ER triggers a Gαq, Gαi, and Gβγ-dependent activation of MMP-2 and -9, mediated through Src activation. By antisense studies, MMP-2 and MMP-9 were shown to be necessary for E_2-induced HB-EGF cleavage and liberation, the transactivation of EGFR, and downstream signaling to ERK and PI3K in breast cancer cells, and p38 MAPK in endothelial cells. It is possible that GPR30 may complex with and mediate membrane ER cross-talk to EGFR. However, recent studies from Ahola *et al.* (82) have called this idea into question. These investigators found that antisense inhibition of endogenous GPR-30 had no effect on E_2 signaling to cell proliferation in MCF-7 cells. Thus, this definitive approach suggests that GPR30 is not required and supports previous studies that ER 1) directly associates with and activates G proteins, and 2) this leads to downstream signaling (71, 72). The molecules involved in the ER-to-EGFR cross-talk are shown in Fig. 2.

The full extent of membrane-initiated signaling by E_2/ER and its dependence on EGFR remains to be defined, and the *in vivo* significance is incompletely understood. However, it was demonstrated more than 10 yr ago, that EGF antibody prevents E_2-induced vaginal and uterine growth (29), implying that cross-talk from ER to the EGFR at the membrane may be physiologically important. Recent studies concerning the role of E_2/ER signaling at the membrane support this idea. Simoncini *et al.* (83) showed that in endothelial cells, ERα directly associates with the membrane-tethered p85 subunit of PI3K. E_2 rapidly activates this kinase, leading to the generation of nitric oxide, and the rescue of rats from ischemia-reperfusion injury of their muscle. It is known that EGFR and PI3K associate (84), and so it is possible that a multiprotein complex exists between ER/PI3K/EGFR and endothelial nitric oxide synthase molecules, perhaps scaffolded onto caveolin-1 at the membrane (19, 21, 85). Similarly, Migliaccio *et al.* (79) showed that ER and Src form a complex. The interaction between ER and Src may be mediated by a newly described adapter protein, modulator of nongenomic activity of estrogen receptor (86). Src activation by E_2 leads to a kinase cascade resulting in ERK activation and DNA synthesis in cancer cells (79). Interestingly, EGFR and Src associate, and both molecules also form complexes

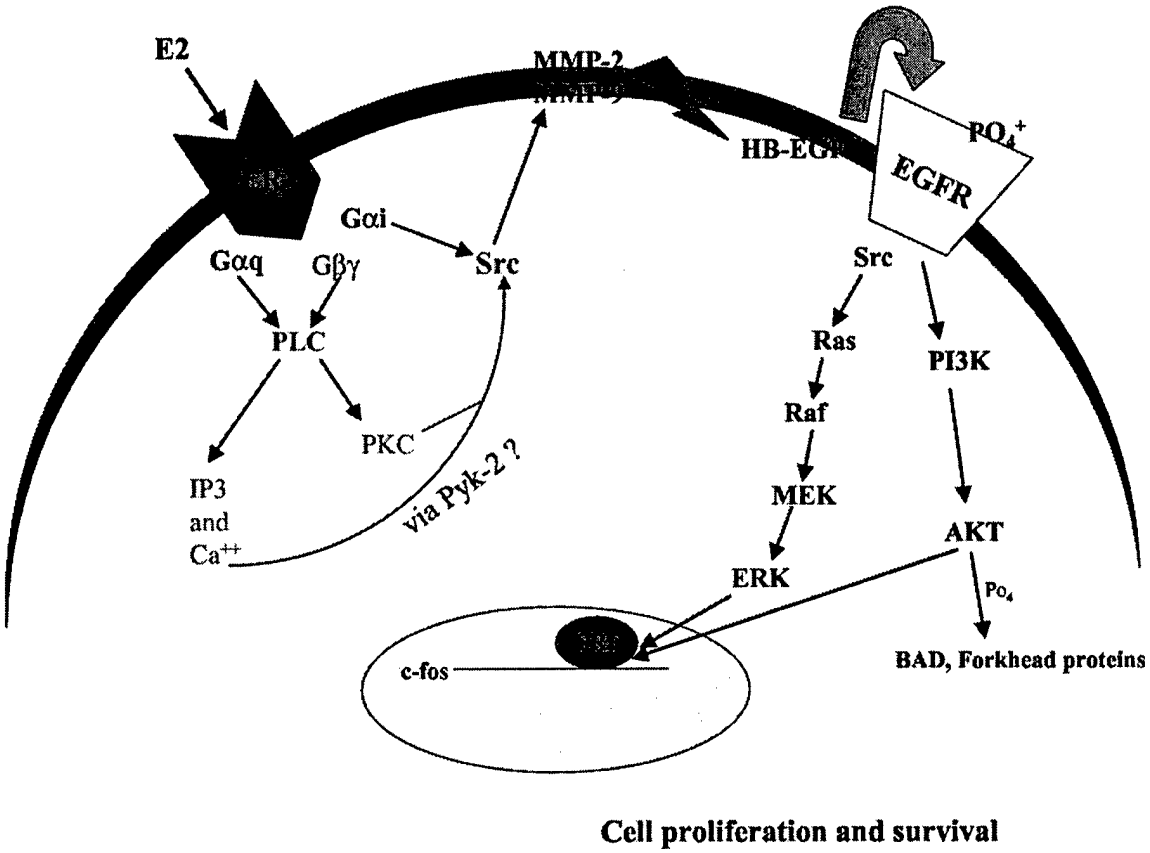


Fig. 2. Membrane ER Cross-Talk to EGFR Leads to Downstream Signaling and Changes in Cell Biology of Breast Cancer

with caveolin-1 (87). Src or EGFR phosphorylates caveolin-1 at the important tyrosine 14, and this leads to the down-regulation of signaling (88). The ability of E_2/ER at the membrane to signal to ERK (via the demonstrated EGFR transactivation) has additional importance for cell biology. Song *et al.* (89) recently demonstrated that ERα lacking a nuclear localization signal and targeted to the plasma membrane activates ERK and cell proliferation in Chinese hamster ovary cells. Also, the survival of breast cancer cells that are subjected to radiation or taxol chemotherapy is enhanced by E_2, partially through ERK activation (90). In aggressive breast cancer, a truncated MTA1 protein was recently found to be highly expressed (91). This protein sequesters ER away from the nucleus and strongly reduces E_2-activated transcription, yet promotes increased ERK signaling and aggressive behavior of the tumor. In neurons subjected to several inducers of apoptosis, E_2 protects these cells through ERK activation (92). The actions of E_2 mediated by this MAPK occur through both protein phosphorylation (90) and gene transcription (93, 94). Most recently, bone loss *in vivo* was prevented by a compound (4-estren-3α, 17β-diol) that has no direct transcriptional activities but activates ERK signaling (95). Therefore, it is probable that the cross-talk from membrane ER through EGFR to downstream kinase activation is biologically important.

The precise structural aspects of the membrane ER that are required for G protein activation are unclear at present but appear to mainly reside in the E domain. This conclusion is based upon the observations that targeting the E domain alone to the plasma membrane allows E_2 activation of ERK (19) and rescues bone cells from an apoptotic cell death (20). Similarly, sending the E domain to the plasma membrane of ER-negative, breast cancer cells results in E_2-induced, Src-dependent matrix metalloproteinase activation, HB-EGF liberation, and EGFR transactivation (78). Thus, the membrane E domain alone can recapitulate the key elements of the pathway from ER to EGFR. These findings are supported by the earlier observation that Src complexes with (and is activated by) E_2/ER, and that tyrosine 537 within the E domain is an essential structural component (96). This may be important for specific signaling pathways, however. Bjornstrom and Sjoberg (97) have recently examined the E_2 rapid activation of signal transducer and activator of transcription (STAT) transcription factor-induced β-casein promoter activation. STAT activation requires both ERK and PI3K, induced by E_2/ER. These authors report that mutating tyrosine 541 of the mouse ERα (equivalent of

human ERα tyrosine 537) has no effect on E_2 induction of the STAT-β-casein pathway. Also, Song *et al.* (89) recently showed that the Src homology 2-domain containing adapter protein, Shc, complexes with ERα through the AF-1 domain, and suggested that this interaction may underlie E_2-induced ERK. However, we recently found that expression of only the membrane-targeted E domain (19), or A/B domain-deleted ERα (unpublished observations) 1) fully binds steroid at the membrane, and 2) comparably activates ERK, compared with expressed wild-type ERα. Thus, current data support a unique and complete role for the E domain in effecting signal transduction initiated at the membrane.

PERSPECTIVE

The bidirectional cross-talk between ER and the growth factor receptors EGFR and IGF-IR indicates a potent method of augmenting E_2 or growth factor action. In a particular cell type and situation, there may be a predominant contribution from one of these pathways, essential to the cell biology of breast cancer, for instance. Tamoxifen is effective in preventing the reoccurrence of ER-positive breast cancer, in part because it inhibits aspects of E_2 and EGFR signaling. In ER-negative breast cancer, there is possibly less restraint on EGFR signaling to cell proliferation or survival in the absence of ER antagonism, thereby contributing to a more aggressive phenotype. Interestingly, in human breast cancer, ER and EGFR concentrations are inversely correlated (98, 99), and ER appears to repress the EGFR gene through a first intron sequence (98). Increased EGFRs in ER-negative breast cancer may also contribute to the more active growth and invasive behavior of these tumors.

The interactions of ER and EGFR impact both the transcriptional and nontranscriptional effects of steroid hormones and protein growth factors, but these are not mutually exclusive actions. Membrane E_2/ER activates PI3K signaling via EGFR (78). As shown by DNA microarray, PI3K activation by E_2 leads to the up-regulation of 250 genes after just 40 min of exposure of endothelial cells to sex steroid (100). Thus, ER-EGFR cross-talk at the membrane enacts multiple signaling pathways that likely have a profound impact on the transcriptional effects of E_2. It is certainly possible that manipulating the specific pathways that result from the bidirectional signaling will yield therapeutic interventions for human disorders that result from excessive growth factor and steroid hormone action.

Acknowledgments

I regret that many fine contributions to this scientific area could not be recognized due to space limitations.

Received November 6, 2002. Accepted December 6, 2002.

Address all correspondence and requests for reprints to: Ellis R. Levin, M.D., Medical Service (111-I), Long Beach Veterans Affairs Medical Center/University of California-Irvine, 5901 East 7th Street, Long Beach, California 90822. E-mail: ellis.levin@med.va.gov.

This work was supported by grants from the Research Service of the Department of Veteran's Affairs, Avon Products Breast Cancer Research Foundation, Department of Defense Breast Cancer Research Program (Grant BC990915), and the NIH (Grant HL-59890).

REFERENCES

1. Wilkie AO, Patey SJ, Kan SH, Van Den Ouweland AM, Hamel BC 2002 FGFs, their receptors, and human limb malformations: clinical and molecular correlations. Am J Med Genet 112:266–278
2. Sledge Jr GW 2002 Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. Semin Oncol 29(Suppl 11):104–110
3. Fant ME, Weisoly D 2001 Insulin and insulin-like growth factors in human development: implications for the perinatal period. Semin Perinatol 25:426–435
4. Betsholtz C, Karlsson L, Lindahl P 2001 Developmental roles of platelet-derived growth factors. Bioessays 23: 494–507
5. Pawson T, Gish GD, Nash P 2001 SH2 domains, interaction modules and cellular wiring. Trends Cell Biol 11:504–511
6. Yaffe MB 2002 Phosphotyrosine-binding domains in signal transduction. Nat Rev Mol Cell Biol 3:177–186
7. Datta SR, Brunet A, Greenberg ME 1999 Cellular survival: a play in three Akts. Genes Dev 13:2905–2927
8. Brunet A, Datta SR, Greenberg ME 2001 Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297–305
9. Formstecher E, Ramos JW, Fauquet M, Calderwood DA, Hsieh JC, Canton B, Nguyen XT, Barnier JV, Camonis J, Ginsberg MH, Chneiweiss H 2001 PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. Dev Cell 1:239–250
10. Watanabe G, Lee RJ, Albanese C, Rainey WE, Batlle D, Pestell RG 1996 Angiotensin II activation of cyclin D1-dependent kinase activity. J Biol Chem 271: 22570–22577
11. Weber JD, Raben DM, Phillips PJ, Baldassare JJ 1997 Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem J :326:61–32668
12. Babu GJ, Lalli MJ, Sussman MA, Sadoshima J, Periasamy M 2000 Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. J Mol Cell Cardiol 32: 1447–1457
13. Kerkhoff E, Houben R, Loffler S, Troppmair J, Lee JE, Rapp UR 1998 Regulation of c-myc expression by Ras/Raf signalling. Oncogene 16:211–216
14. White, R and Parker MG 1998 Molecular mechanisms of steroid hormone action. Endocr Relat Cancer 5:1–14
15. Sukovich DA, Mukherjee R, Benfield PA 1994 A novel, cell-type-specific mechanism for estrogen receptor-mediated gene activation in the absence of an estrogen-responsive element. Mol Cell Biol 14:7134–7143
16. Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 264:1455–1458
17. Falkenstein E, Wehling M 2000 Nongenomically initiated steroid actions. Eur J Clin Invest 30:51–54

18. Zhang Z, Maier B, Santen RJ, Song RX 2002 Membrane association of estrogen receptor α mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun 294:926–933
19. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER 2002 Estrogen receptors associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol Endocrinol 16:100–115
20. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719–730
21. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW 2000 Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res 87:e44–e52
22. Alroy I, Yarden Y 1997 The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett 410:83–86
23. Schlessinger J 2000 Cell signaling by receptor tyrosine kinases. Cell 103:211–225
24. Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ 2001 Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. J Biol Chem 276:23155–23160
25. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F 1998 Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross talk with estrogen receptor. EMBO J 17:2008–2018
26. Riesell DJ, Stern DF 1998 Specificity within the EGF family/ErbB receptor family signaling networks. Bioessays 20:41–48
27. Mukku VR, Stancel GM 1985 Regulation of epidermal growth factor receptor by estrogen. J Biol Chem 260: 9820–9824
28. DiAugustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA, Teng CT 1988 Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. Endocrinology 122:2355–2363
29. Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan JA 1991 Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. Proc Natl Acad Sci USA 88:21–25
30. Dickson RB, Huff KK, Spencer EM, Lippman ME 1986 Induction of epidermal growth factor-related polypeptides by 17 β-estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142
31. Hom YK, Young P, Wiesen JF, Miettinen PJ, Derynck R, Werb Z, Cunha GR 1998 Uterine and vaginal organ growth requires epidermal growth factor receptor signaling from stroma. Endocrinology 139:913–921
32. Vignon F, Bouton MM, Rochefort H 1987 Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. Biochem Biophys Res Commun 146:1502–1508
33. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS 1992 Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci USA 89:4658–4662
34. Huang HJ, Norris JD, McDonnell DP 2002 Identification of a negative regulatory surface within estrogen receptor α provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. Mol Endocrinol 16:1778–1792
35. Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF, Korach KS 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc Natl Acad Sci USA 93:12626–12630
36. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige X, Gotoh Y, Nishida E, Kawashima H 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491–1494
37. Bunone G, Briand PA, Miksicek RJ, Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174–2183
38. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 7:992–998
39. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS 1996 Peptide growth factor cross talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology 137:1735–1744
40. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, Lannigan DA 1998 pp90rsk1 Regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol 18:1978–1984
41. Chen D, Washbrook E, Sarwar N, Bates GJ, Pace PE, Thirunuvakkarasu V, Taylor J, Epstein RJ, Fuller-Pace FV, Egly JM, Coombes RC, Ali S 2002 Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. Oncogene 21:4921–4931
42. Lee H, Bai W 2002 Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol Cell Biol 22:5835–5845
43. Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivio SE, Stoica A 2000 A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinology 141:4503–4511
44. Wang, R-A, Mazumdar, A, Vadlamudi RK, Kumar R 2002 P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-α and promotes hyperplasia in mammary epithelium. EMBO J 21:5437–5447
45. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ 2001 Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 276: 22177–22182
46. Feng W, Webb P, Nguyen P, Liu X, Li J, Karin M, Kushner PJ 2001 Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. Mol Endocrinol 15:32–45
47. Wiepz GJ, Houtman JC, Cha D, Bertics PJ 1997 Growth hormone attenuation of epidermal growth factor-induced mitogenesis. J Cell Physiol 173:44–53
48. Zwijsen RM, Wientjens E, Klompmaker R, van der Sman J, Bernards R, Michalides RJ 1997 CDK-independent activation of estrogen receptor by cyclin D1. Cell 88: 405–415
49. Zwijsen RM, Buckle RS, Hijmans EM, Loomans CJ, Bernards R 1998 Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Genes Dev 12:3488–3498
50. McMahon C, Suthiphongchai T, DiRenzo J, Ewen ME 1999 P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc Natl Acad Sci USA 96:5382–5387

51. Hewitt SC, Bocchinfuso WP, Zhai J, Harrell C, Koonce L, Clark J, Myers P, Korach KS 2002 Lack of ductal development in the absence of functional estrogen receptor α delays mammary tumor formation induced by transgenic expression of ErbB2/neu. Cancer Res 62: 2798–2805
52. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L, Hung MC 2001 Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 3:802–808
53. Cordero JB, Cozzolino M, Lu Y, Vidal M, Slatopolsky E, Stahl PD, Barbieri MA, Dusso A 2002 A 1,25-dihydroxyvitamin D down-regulates cell membrane growth- and nuclear growth-promoting signals by the epidermal growth factor receptor. J Biol Chem 277:38965–38971
54. Klotz DM, Hewitt SC, Ciana P, Raviscioni M, Lindzey JK, Foley J, Maggi A, DiAugustine RP, Korach KS 2002 Requirement of estrogen receptor-α in insulin-like growth factor-1 (IGF-1)-induced uterine responses and *in vivo* evidence for IGF-1/estrogen receptor cross talk. J Biol Chem 277:8531–8537
55. Varma H, Conrad SE 2002 Antiestrogen ICI 182,780 decreases proliferation of insulin-like growth factor I (IGF-I)-treated MCF-7 cells without inhibiting IGF-I signaling. Cancer Res 62:3985–3991
56. Oesterreich S, Zhang P, Guler RL, Sun X, Curran EM, Welshons WV, Osborne CK, Lee AV 2001 Re-expression of estrogen receptor α in estrogen receptor α-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. Cancer Res 61:5771–5777
57. Chan TW, Pollak M, Huynh H 2001 Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. Clin Cancer Res 7:2545–2554
58. Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, Yee D 1999 Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. Mol Endocrinol 13:787–796
59. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C 2000 Estrogen receptor α rapidly activates the IGF-1 receptor pathway. J Biol Chem 275:18447–18453
60. Dupont J, Karas M, LeRoith D 2000 The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. J Biol Chem 275:35893–359901
61. Lai A, Sarcevic B, Prall OW, Sutherland RL 2001 Insulin/insulin-like growth factor-I and estrogen cooperate to stimulate cyclin E-Cdk2 activation and cell cycle progression in MCF-7 breast cancer cells through differential regulation of cyclin E and p21(WAF1/Cip1). J Biol Chem 276:25823–25833
62. Sato T, Wang G, Hardy MP, Kurita T, Cunha GR, Cooke PS 2002 Role of systemic and local IGF-I in the effects of estrogen on growth and epithelial proliferation of mouse uterus. Endocrinology 143:2673–2679
63. Tesarik J, Mendoza C 1995 Nongenomic effects of 17β-estradiol on maturing human oocytes: relationship to oocyte developmental potential. J Clin Endocrinol Metab 80:1438–1443
64. Kelly MJ, Wagner EI 1999 Estrogen modulation of G-protein coupled receptors. Trends Endocrinol Metab 10:369–374
65. Aronica SM, Kraus WL, Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci USA 91:8517–8522
66. Le Mellay V, Grosse B, Lieberherr M 1997 Phospholipase C β and membrane action of calcitriol and estradiol. J Biol Chem 272:11902–11907
67. Lieberherr M, Grosse B, Kachkache M, Balsan S 1993 Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional non-nuclear receptors. J Bone Miner Res 8:1365–1376
68. Kelly MJ, Levin ER 2001 Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 12: 152–156
69. Pietras R, Szego CM 1977 Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265:69–72
70. Norfleet AM, Thomas ML, Gametchu B, Watson CS 1999 Estrogen receptor-α detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. Endocrinology 140:3805–3814
71. Razandi M, Pedram A, Greene GL, Levin ER 1999 Cell membrane and nuclear estrogen receptors derive from a single transcript: studies of ERα and ERβ expressed in CHO cells. Mol Endocrinol 13:307–319
72. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Gα(i). J Biol Chem 276:27071–27076
73. Daub H, Weiss FU, Wallasch C, Ullrich A 1996 Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 379:557–560
74. Filardo EJ, Quinn JA, Bland KI, Frackelton Jr AR 2000 Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, gpr30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14:1649–1660
75. Filardo EJ, Quinn JA, Frackelton Jr AR, Bland KI 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16: 70–84
76. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A 1999 EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402:884–888
77. Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ 1997 Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45:607–617
78. Razandi M, Pedram A, Parks S, Levin ER 2003 Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem 278:2701–2712 (First published 5 November 2002; 10.1074/jbc.M205692200)
79. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F 2000 Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation. EMBO J 19:5406–5417
80. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR 2000 Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci USA 97: 5930–5935
81. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, Gustafsson JA, Mendelsohn ME 2002 Abnormal vascular function and hypertension in mice deficient in estrogen receptor β. Science 295:505–508
82. Ahola TM, Manninen T, Alkio N, Ylikomi T 2002 G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology 143:3376–3384

83. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541
84. Yamabhai M, Anderson RG 2002 Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. J Biol Chem 277:24843–24846
85. Okamoto T, Schlegel A, Scherer PE, Lisanti MP 1998 Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J Biol Chem 273:5419–5422
86. Wong, C-W, McNally C, Nickbarg E, Komm BS, Cheskis BJ 2002 Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. Proc Natl Acad Sci USA 99:14783–14788
87. Lee H, Volonte D, Galbiati F, Iyengar P, Lublin DM, Bregman DB, Wilson MT, Campos-Gonzalez R, Bouzahzah B, Pestell RG, Scherer PE, Lisanti MP 2000 Constitutive and growth factor-regulated phosphorylation of caveolin-1 occurs at the same site (Tyr-14) *in vivo*: identification of a c-Src/Cav-1/Grb7 signaling cassette. Mol Endocrinol 14:1750–1775
88. Park WY, Cho KA, Park JS, Kim DI, Park SC 2001 Attenuation of EGF signaling in senescent cells by caveolin. Ann NY Acad Sci 928:79–84
89. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ERα-Shc association and Shc pathway activation. Mol Endocrinol 16:116–127
90. Razandi M, Pedram A, Levin ER 2000 Plasma membrane estrogen receptors signal to anti-apoptosis in breast cancer. Mol Endocrinol 14:1434–1447
91. Kumar R, Wang E-A, Mazumdar A, Talukder AH, Mandal M, Yang Z, Bagheri-Yarmand R, Sahin A, Hortobagyi G, Adam L, Barnes CJ, Vadlamudi RK 2002 A naturally occurring MTA1 variant sequesters oestrogen receptor-a in the cyctoplasm. Nature 418:654–657
92. Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM 1999 The mitogen activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J Neurosci 19: 2455–2463
93. Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM 1997 Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology 138: 4030–4033
94. Watters JJ, Chun TY, Kim YN, Bertics PJ, Gorski J 2000 Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. Mol Endocrinol 14:1872–1881
95. Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC 2002 Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 298:843–846
96. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F 1996 Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15: 1292–1300
97. Bjornstrom L, Sjoberg M 2002 Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. Mol Endocrinol 16:2202–2214
98. Wilson MA, Chrysogelos SA 2002 Identification and characterization of a negative regulatory element within the epidermal growth factor receptor gene first intron in hormone-dependent breast cancer cells. J Cell Biochem 85:601–614
99. deFazio A, Chiew YE, Sini RL, Janes PW, Sutherland RL 2000 Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. Int J Cancer 87:487–498
100. Pedram A, Razandi M, Aitkenhead M, Hughes CCW, Levin ER 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 277:50768-50775 (First published 7 October 2002; 10.1074/jbc.M210106200)

MOLECULAR AND CELLULAR BIOLOGY, Mar. 2003, p. 1633–1646 Vol. 23, No. 5
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.5.1633–1646.2003

Identification of a Structural Determinant Necessary for the Localization and Function of Estrogen Receptor α at the Plasma Membrane

Mahnaz Razandi,[1,2] Gordon Alton,[3] Ali Pedram,[1,2] Sanjiv Ghonshani,[1,2] Paul Webb,[4] and Ellis R. Levin[1,2,5]*

Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822[1]; Departments of Medicine[2] and Pharmacology,[5] University of California, Irvine, Irvine, California 92717; Signal Pharmaceuticals Inc., San Diego, California 92121[3]; and Diabetes and Metabolic Research Unit at the University of California, San Francisco, San Francisco, California 94143[4]

Received 19 July 2002/Returned for modification 29 August 2002/Accepted 6 December 2002

Estrogen receptors (ER) have been localized to the cell plasma membrane (PM), where signal transduction mediates some estradiol (E2) actions. However, the precise structural features of ER that result in membrane localization have not been determined. We obtained a partial tryptic peptide/mass spectrometry analysis of membrane mouse ERα protein. Based on this, we substituted alanine for the determined serine at amino acid 522 within the E domain of wild-type (wt) ERα. Upon transfection in CHO cells, the S522A mutant ERα resulted in a 62% decrease in membrane receptor number and reduced colocalization with caveolin 1 relative to those with expression of wt ERα. E2 was significantly less effective in stimulating multiple rapid signals from the membranes of CHO cells expressing ERα S522A than from those of CHO cells expressing wt ERα. In contrast, nuclear receptor expression and transcriptional function were very similar. The S522A mutant was also 60% less effective than wt ERα in binding caveolin 1, which facilitates ER transport to the PM. All functions of ERα mutants with other S-to-A substitutions were comparable to those of wt ER, and deletion of the A/B or C domain had little consequence for membrane localization or function. Transfection of ERα S522A into breast cancer cells that express native ER downregulated E2 binding at the membrane, signaling to ERK, and G_1/S cell cycle events and progression. However, there was no effect on the E2 transactivation of an ERE-luciferase reporter. In summary, serine 522 is necessary for the efficient translocation and function of ERα at the PM. The S522A mutant also serves as a dominant-negative construct, identifying important functions of E2 that originate from activating PM ER.

Steroid action is attributed primarily to the regulation of target genes through nuclear receptor binding and transactivation, subsequently producing cell biological effects (42). However, it has increasingly been appreciated that steroids, such as estradiol (E2), act rapidly through nongenomic mechanisms of signal transduction (4, 16, 41). These signaling mechanisms have important consequences for the effects of steroids on cell biology (14, 40). For E2, these effects can occur after the sex steroid binds to plasma membrane (PM) estrogen receptors (ER) (17, 30), which has been demonstrated by immunohistochemistry (36) and by immunoblotting of isolated PM domains (6). Some signaling effects of E2-ER can result from complex interactions with PM growth factor tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) (10).

Although the exact sequence of this receptor has not been reported, the membrane ER appears to be very similar, and perhaps identical, to the nuclear receptor. This is based upon the identification of similarly sized nuclear and membrane ER proteins that result from the expression of a single cDNA (and resulting single mRNA) in CHO cells (32). Also, membrane ER have been localized on vascular smooth muscle, pituitary, and endothelial cells that express endogenous receptors, by using antibodies raised against multiple epitopes of the nuclear ERα (25, 26, 36). However, many questions remain concerning this relatively small population of ER at the cell surface.

The membrane ER has been reported to be G protein linked (32, 50), and E2 binding can activate many signal transduction pathways that emanate from G protein activation. These include kinase and endothelial nitric oxide synthase activation, cyclic AMP (cAMP) and inositol phosphate (IP) generation, and phospholipase C (PLC) stimulation (4, 16, 18, 24, 50). Linkage to G proteins may be direct, as shown in transfected CHO cells expressing ERα or ERβ (32) or in endothelial cells (50), but it has also been reported that E2 activates an orphan G protein-coupled receptor (10). Furthermore, it is not clear whether this receptor spans the cell membrane or is predominately localized within or associated with the membrane bilayer. Membrane ER have recently been shown to exist in discrete caveolar domains of the PM (6, 13). It has recently been found that membrane ERα can physically associate with the caveolar structural coat proteins caveolin 1 and caveolin 2 (31). Caveolin proteins serve as scaffolds, bringing together various signaling molecules within a discrete area of the PM to regulate cytokine-induced signal transduction (3, 27). These include G proteins, nonreceptor and receptor tyrosine kinases (Src, EGFR), and threonine-serine kinases, such as phospha-

* Corresponding author. Mailing address: Medical Service (111-I), Long Beach VA Medical Center, 5901 E. 7th St., Long Beach, CA 90822. Phone: (562) 826-5748. Fax: (562) 826-5515. E-mail: ellis.levin@med.va.gov.

tidylinositol 3-kinase (PI 3-kinase) and Raf. Organization of signaling molecules within a confined space potentially allows E2-ER to modulate a variety of signaling cascades in target cells.

Signal transduction via the membrane ER has increasingly been found to be important for the cell biological effects of this steroid, including the survival and/or growth of breast cancer, bone, and neural cells (5, 7, 14, 24, 34, 49). This receptor has also been implicated in prevention of the inflammatory response to muscle ischemia-reperfusion injury (40), maintenance of the endothelial cell cytoskeleton, and upregulation of vascular cell migration and angiogenesis (33). E2 stimulation of transcription can also be signal dependent, as stimulation of the mitogen-activated protein (MAP) kinase ERK (extracellular regulated kinase) has been shown to be important for transactivation of the c-*fos* and prolactin genes (9, 45, 46). Transcription in response to E2 generation of cAMP has also been reported (4). The precise structural features of ER that facilitate the translocation of this steroid binding protein to the membrane are not known, but such information is important for understanding of the details of estrogen action at the cell surface.

The studies reported here result from our attempt to understand the localization and function of this protein at the PM. To begin this, we partially determined the amino acid structure of the mouse membrane ERα, isolated from CHO cells transfected to express this protein. We identified a serine residue at 522 that is necessary for the optimal localization and function of the sex steroid receptor at the cell surface. In contrast, mutation of this serine had no effect on nuclear ER number, affinity for E2, or E2-induced transactivation function. We also report that expression of the S522A mutant ERα resulted in a dominant-negative action only at the membrane, in cells expressing wild-type (wt) ERα. This mutant abolished several important effects of E2 in breast cancer and can be used as a reagent to deduce the cellular actions of E2 originating from membrane ERα.

MATERIALS AND METHODS

Isolation and partial sequencing of a membrane ER. CHO-K1 cells were transiently transfected with a cDNA for the mouse ERα, as previously described (32). This resulted in the expression of both nuclear and membrane receptors. Twenty plates of ERα-transfected CHO cells were scraped and pelleted at 1,000 × *g*, and pellets were resuspended in 20 mM Tris with 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors. Cells were then centrifuged at 4°C and 8,000 × *g* for collection of nuclear receptors, and the supernatant was then ultracentrifuged at 4°C and 100,000 × *g* for 1 h. The pellet (membranes) was washed and ultracentrifuged again, and membranes were then further separated by sucrose gradient overlay; fractions 3 to 5 contained the buoyant membranes (with caveolae and rafts) that were pooled for all experiments (31). Briefly, membrane samples were first placed in a tube with an equal volume of a solution containing 85% (wt/vol) sucrose, 25 mM A-morpholine-ethanesulfonic acid, and 0.15 M NaCl and were then overlaid with 8.5 ml of 35% sucrose, topped up with 16% sucrose, and centrifuged at 35,000 rpm (105,000 × *g*) for 18 h at 4°C. Ten fractions (1 ml each) were obtained and either further processed or separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by membrane transfer for immunoblotting. The membrane receptors were solubilized in binding buffer (Pierce) containing 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma). The purity of the membranes was confirmed by positive immunoblotting for 5′-nucleotidase and caveolin 1 (membrane proteins) and by the lack of detection of transportin and NTF-2 (nuclear proteins) or β-coatomer protein (endosomal/Golgi protein) (38). This was followed by affinity column purification. Briefly, protein G bound to an antibody against ERα (H222) (11) was cross-linked with disuccinimidyl suberate to make the column. The ERα-containing membrane or nuclear protein was eluted by using proprietary buffers and a proprietary protocol (Pierce). The eluted receptor proteins were dialyzed or concentrated and then analyzed by SDS-PAGE after being run on a 7.5% gel followed by staining. The gel protein bands corresponding to 67 kDa were cut out, trypsin was extracted from the gel, and the bands were then subjected to peptide degradation-mass spectrometry, as previously described (2, 12). This generated peptide sequences from the membrane and nuclear proteins, and these were compared to the known sequences of the classical mouse nuclear ERα.

Site directed mutagenesis and targeting of mouse ERα. We carried out tryptic peptide matrix-assisted laser desorption ionization (MALDI)-mass spectrometry "sequencing," as described above. At present this has yielded membrane peptide sequences that identically overlap with 20% of the known nuclear receptor and with 20% of our expressed nuclear receptor, supporting the idea that the two receptors are the same (G. Alton, M. Razandi, A. Pedram, and E. Levin, unpublished data). We identified an overlap sequence from amino acid 508 to amino acid 524 that includes a serine at 522. With surrounding residues, this was identified by computer analysis as a potential (although not a classic) palmitoylation site (HMSN). This sequence is present as amino acids 517 to 520 of the human receptor as well. We then mutated the serine at 522 to alanine in mouse pcDNA3-ERα by PCR using the forward primer 5′-CGGCACATGGCTAACA AAGG-3′. As specificity controls, we also mutated the identified serine residues 10 and 582 to alanine by using the forward primers 5′-CCCTTCACACCAAA GCCGCGGGAATGGCCTTGCTGC-3′ and 5′-GCTCCACTTCAGCACATG CCTTACAAACCTACTAC-3′, respectively. All mutations were confirmed by sequencing at the University of California—Irvine sequencing facility. We additionally subcloned the receptors into a green fluorescent protein (GFP) vector, pEGFPc2 (Clontech, Palo Alto, Calif.), and a multicopy histidine-expressing vector to monitor transfection efficiencies. wt and mutant receptor expression plasmids were then used in studies. To generate nuclear and membrane wt ERα constructs, pcDNA3-mouse ERα was used as a template. Twenty-five cycles of PCR (annealing temperature, 55°C) were performed by utilizing the forward primer 5′GCCGCTAGCACCATGACCATGACCCTTCAC3′ and the lower primer 5′GCCACCGGTCTGATCGTGTTGGGGAAGCCC3′. The PCR product was ligated into pCR2.1 by using the TA cloning kit (Invitrogen, Carlsbad, Calif.) and digested with *Age*I and *Nhe*I. This fragment was subcloned into *Age*I and *Nhe*I sites on the pECFP-Nuc and pECFP-mem vectors (Clontech), yielding ER constructs that were predominantly targeted to either the membrane or the nucleus (confirmed by binding and functional studies).

Receptor binding and cell localization studies. wt and mutant ERα were expressed in CHO cells, and nuclear and membrane fractions were isolated as detailed above and were used for competitive binding assays or signal transduction studies, as previously described (31, 32). Binding studies were repeated at least three times, and the data were used for Scatchard analysis with the LIGAND computer program. Results were combined for statistical comparison by analysis of variance plus Schefe's test. Additional ERα mutants (HE11G, with the A/B domain deleted; HE19G, with the C domain deleted; and HEG0-537, with helix 12 and the F domain deleted) were provided by Paul Webb and expressed in CHO cells.

For cell localization of wt or S522A mutant ERα, we transiently expressed GFP-tagged fusion proteins for each receptor in CHO cells. CHO cells were grown and transfected on coverslips, and localization of the receptors was examined by laser-scanning confocal microscopy. We also colocalized the receptors at the membrane with endogenous caveolin 1 by using an antibody to this protein (Zymed Laboratories, South San Francisco, Calif.). Each section was processed for GFP-ERα (green), caveolin 1 (second antibody conjugated to Texas red), and colocalized caveolin 1 and ERα (yellow).

Signaling studies. Adenylate cyclase activity in the membrane was determined by measuring cAMP generation, by methods described previously (32), in CHO-K1 cells expressing wt or S522A mutant ERα after the cells had been incubated for 5 min with 10 nM E2. IP generation and ERK (MAP kinase) activation in the CHO cells were also determined as described in detail elsewhere (32). Activation by E2 of an ERE-luciferase reporter in ER-transfected CHO or MCF-7 cells was assessed at 8 h of exposure to 10 nM E2, as previously published (32). Membranes were isolated by sucrose gradient centrifugation (31).

Myristylation, palmitoylation, and PI-PLC studies in CHO-K1 cells. Cells were grown on 100-mm-diameter petri dishes in Dulbecco's modified Eagle medium (DMEM)-F12 medium without phenol red. Twenty-four hours after transfection with ERα, the cells were synchronized overnight and then labeled with [^{3}H]palmitic acid (0.5 μCi/ml) or [^{3}H]myristic acid (0.2 μCi/ml) for 2 h. The cells were incubated for 8 h in the presence or absence of 10 nM 17β-E2, washed with cold phosphate-buffered saline, and then lysed in buffer A (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 100 mM NaCl, 50 mM NaF, 100 μM phenylmethylsul-

fonyl fluoride, protease inhibitor cocktail, and 0.2% Triton X-100). Nuclear pellets were collected by low-speed centrifugation. Supernatants were centrifuged at 100,000 × *g* for 30 min to pellet cell membranes. Both pellets were washed twice, once with buffer A and once without detergent. Membranes were further purified by sucrose gradient centrifugation. Membrane and nuclear fractions were denatured in SDS loading buffer followed by gel electrophoresis, fluorography, and autoradiography. For phosphoinositol (PI)-PLC studies, the cells were incubated with 1 U of PI-PLC (Sigma)/ml for 1 h. Cells were washed and lysed, and the membrane and nuclear fractions were collected. Specific, total binding studies were then carried out on 50 μl of nuclear or membrane protein, incubated in DMEM-F12 medium (with no phenol red), bacitracin (1 mg/ml), and 0.5% bovine serum albumin, and with ^{3}H-labeled E2 and unlabeled E2 (10^{-11} to 10^{-7} M).

Cyclin D1 protein expression, thymidine incorporation, and kinase activity. MCF-7 cells were transfected with pcDNA3 (control) or ERα S522A, recovered, then synchronized by serum deprivation for 24 h, and then incubated in the presence or absence of 10 nM E2 for 8 h. In some cells, the soluble MEK inhibitor PD98059 (10 μM) was added to the incubation mixture 30 min prior to the steroid. The cells were then lysed, precleared, boiled, denatured in SDS reducing buffer, and electrophoretically resolved by PAGE. Western immunoblotting was then carried out using a polyclonal antibody (Santa Cruz). Nuclear thymidine incorporation was carried out in nontransfected or transfected MCF-7 cells after synchronization overnight in serum-free medium. All cells were then incubated for 20 h in the absence or presence of 10 nM 17β-E2. In some conditions, the MEK inhibitor PD 98059 (10 μM) was added prior to the steroid. After 20 h, 0.5 μCi of [^{3}H]thymidine was added for 4 more h, as previously described (32). Cells were then washed and incubated for 10 min with 10% trichloroacetic acid at 4°C, followed by additional washes. Cells were lysed with 0.2 N NaOH overnight, and lysates were counted in a liquid scintillation β-counter. For cdk4 activity, MCF-7 cells transfected with pcDNA3 (control) or ERα S522A were incubated with E2 for 6 h and then lysed. The cell lysate was added to a protein A-Sepharose-conjugated cdk4 antibody (Santa Cruz) and then added to in vitro kinase activity tubes containing GST-pRB as a substrate, as previously described (28). This was followed by SDS-PAGE separation and autoradiography. Samples from each condition were assessed for protein loading equivalence, where cdk4 protein was assayed by immunoblotting. For ERK activity assays, transfected or nontransfected CHO, MCF-7, or ZR-75-1 cells were synchronized for 24 h in serum, phenol red, and growth factor-free medium. The cells were then exposed to E2 (10 nM) for 8 min with or without additional substances, and kinase activity was determined by using myelin basic protein (MBP) for the substrate, as previously described (32, 34). For p38β activity, the cells were incubated with E2 (10 nM) for 20 min and then lysed, and the lysate was immunoprecipitated with protein A-Sepharose conjugated to an antiserum for p38β. Immunoprecipitated kinases were then added to the protein ATF-1 for in vitro kinase assays as previously described (33). All experiments were repeated two to three times.

Protein and ER association studies. Cytosolic fractions of CHO-wt ERα or CHO-ERα S522A were incubated with protein A-Sepharose for 1 h, and supernatants were transferred to fresh tubes containing protein A-Sepharose conjugated to caveolin 1 or ERα antibodies and were incubated for 4 h at 4°C. Immune complexes were washed, boiled, and then separated by SDS-PAGE. After transfer to nitrocellulose filters, the nonspecific proteins were blocked with blocking solution (Bio-Rad) and incubated first with a primary antibody to ERα or caveolin 1 for 2 h and then with a second antibody (Santa Cruz Biotechnology). Bound immunoglobulin G's (IgGs) were visualized using ECL reagents (Amersham) and autoradiography. Portions of the immunoprecipitated ERα or caveolin 1 were immunoblotted for evidence of equal protein loading and equal expression of total ER with the two constructs. In additional studies, MCF-7 cells were transfected to express a GFP-ERα S522A protein or GFP alone. After overnight recovery, the cells were lysed and immunoprecipitated with an antibody to GFP, followed by immunoblotting with antibodies to Src, Ras, and Raf proteins (Santa Cruz). In CHO cells, His-wt ERα or GFP-ERα S522A was singly or doubly expressed. To detect homo- or heterodimerization in these cells, the lysate was immunoprecipitated with an antibody to His, followed by blotting with an antibody to GFP, or in reverse order. All studies were repeated at least three times.

RESULTS

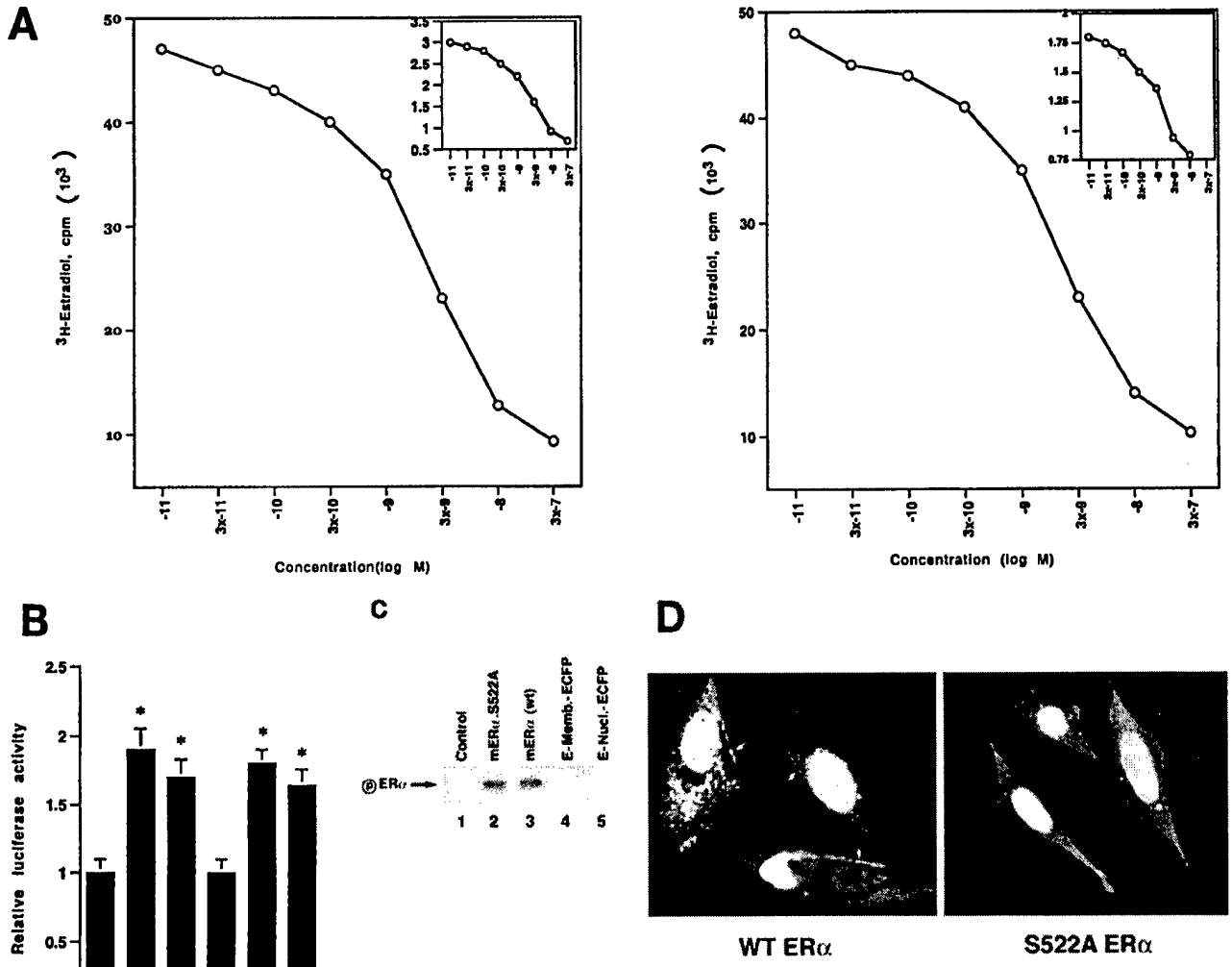
Comparison of wt and S522A mutant ERα binding after expression in CHO cells. We first isolated the mouse ERα in the PM after expression in CHO cells and partially sequenced the protein by peptide degradation-MALDI mass spectroscopy (Razandi et al., unpublished). We identified a peptide (LA QLLLILSHIRHMSNK) that corresponds to a portion of the C terminus in the known mouse ERα sequence (2), beginning with amino acid 508. Furthermore, HMS (boldfaced in peptide sequence) was noted by computer modeling as a possible, but not classic, palmitoylation site (35). We therefore asked whether the ER was palmitoylated at this site (see below), and we also mutated the critical serine at amino acid position 522 to alanine within the mouse ERα cDNA. Additional S-to-A mutations at residues 10 and 582 were created by site-directed mutagenesis, for comparison to mouse ERα S522A (48) and to support the specificity of any findings.

We then expressed the wt and S522A mutant ERα constructs in CHO cells and carried out competitive binding studies in both nuclear and membrane compartments. By Scatchard analysis of the binding data (Fig. 1A), we found that the receptor affinity for E2 (K_d) and the receptor number (B_{max}) were very similar for the two ERα receptors in the nucleus (Table 1). Similar transfection efficiencies were demonstrated using GFP fusion constructs (data not shown). We also determined whether the function of the mutant nuclear ERα differed from that of the wt. We therefore cotransfected CHO cells with either wt or S522A mutant ERα and an ERE-luciferase reporter (32) and determined the response to E2. We found that the two receptors were comparably capable of responding to E2 with an upregulation of reporter activity (Fig. 1B). These data indicate that the replacement of S with A at residue 522 does not affect the quantity of nuclear receptor localization, its binding affinity for E2, or the transcriptional response to the steroid.

In contrast, binding experiments revealed that expression of the S522A mutant ERα resulted in 62% fewer receptors (B_{max}) in the PM than expression of wt ERα (Fig. 1A insets; Table 1). This was found in three separate binding experiments, where the reduction ranged from 57 to 65%. The binding affinities (K_d) for E2 at the membrane were comparable for wt and mutant receptors. Thus, serine 522 is an important determinant for full membrane localization of ERα.

It is possible that serine 522 is a phosphorylation site, although this would not be a common mechanism for membrane localization. By mass spectroscopy, there was no evidence of phosphorylation on this residue. We also expressed the full-length wt ER, the S522A mutant, or the E domain (ligand binding domain) of wt ERα in CHO cells, targeting the E domain to both nuclear and membrane locations. As seen in Fig. 1C, the entire receptor is phosphorylated at serine/threonine residues, but we find no evidence that the intact E domain is similarly phosphorylated, either when targeted to the membrane or when targeted to the nucleus.

To visualize the receptor at the membrane, we expressed GFP-tagged wt or S522A mutant ERα in CHO cells and detected membrane localization by confocal microscopy. As seen in Fig. 1D, wt receptor expression clearly reveals a population of membrane-localized sex steroid binding proteins, while the mutant receptor does not. Both show a dense nuclear population. We also examined colocalization of the wt or mutant ERα at the membrane with caveolin 1. In Fig. 1E, wt ERα was clearly seen at the membrane (arrow, panel A), in contrast to sparse membrane expression of ERα S522A (panel D). Caveo-


A
^{3}H-Estradiol, cpm (10^3)
Concentration(log M)
Concentration (log M)
B
Relative luciferase activity
mERa (wt)
+ E2 10nM
+ E2 1nM
mERa.S522A
+ E2 10nM
+ E2 1nM
C
Control
mERα.S522A
mERα (wt)
E-Memb.-ECFP
E-Nucl.-ECFP
pERα
1 2 3 4 5
D
WT ERα
S522A ERα
E
ERα
Caveolin-1
Merge
WT-ERα
S522A-ERα
A
B
C
D
E
F

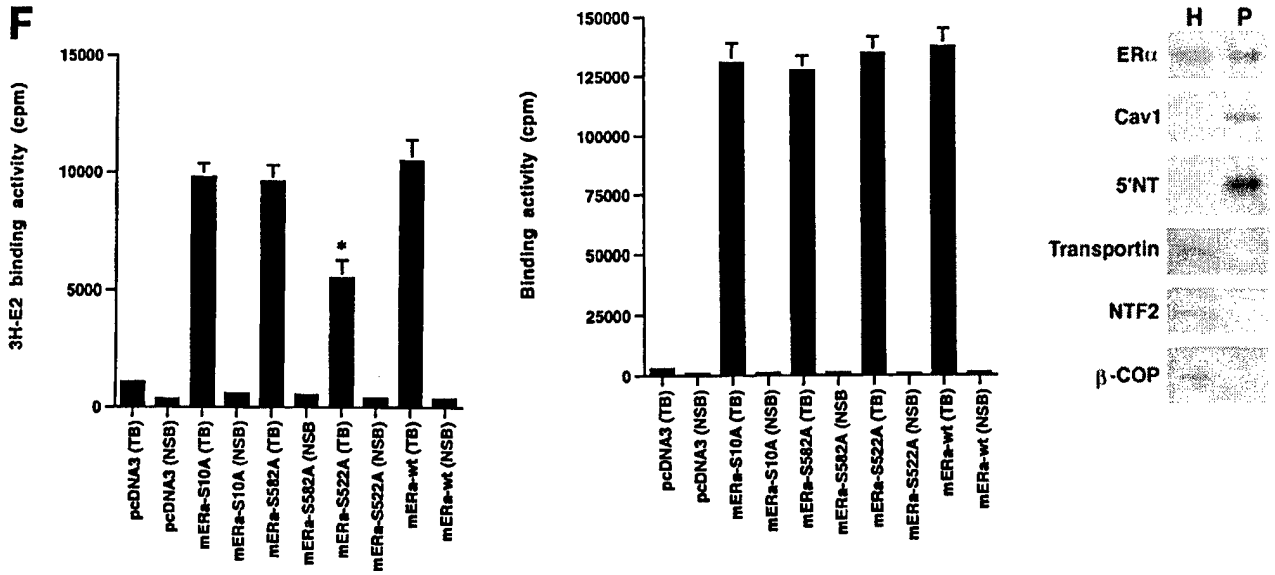


FIG. 1. (A) Competition binding of 17β-[^{3}H]E2 to nuclear wt (left) or S522A mutant (right) ERα transfected into CHO-K1 cells. (Inset) Binding to cell membrane ERα. Data are transformed for Scatchard analysis by using the LIGAND program. Data shown here are from a representative study; results from three separate experiments were combined to create Table 1. (B) Transactivation of an ERE-luciferase reporter construct coexpressed in CHO cells with mouse wt ERα or the S522A mutant. Data were determined at 6 h after incubation with either 1 nM E2, 10 nM E2, or no steroid. *, $P < 0.05$ for the wt or the S522A mutant alone versus the same construct plus E2 (for data combined from three experiments). (C) Serine/threonine residues in the full-length receptor, but not serine 522 in the E domain of ERα, are phosphorylated. Western blotting utilized a specific antibody to serine/threonine residues (Sigma) from lysates of CHO cells transfected to express either the full-length receptor or the E domain targeted to the PM or the nucleus. (D) Membrane localization of GFP-tagged wt ERα or S522A mutant ERα expressed in CHO cells. Arrows indicate a greater membrane localization for wt ERα. Dense nuclear populations for both receptors are seen. (E) Colocalization of wt ERα with caveolin 1 at the membrane, but markedly less colocalization of ERα S522A. Results of a representative study are shown. Arrows indicate differential ER expression (green) at the membrane (panels A and D) and equal caveolin 1 expression (red) (panels B and E). The strong colocalization of wt ERα and caveolin 1 (yellow) (arrow, panel C) is not seen for ERα S522A (arrow, panel F). (F) Total specific binding of labeled E2 to membranes (left) or nuclei (center) in CHO cells expressing either S10A, S582A, or S522A mutant ERα or wt ERα. Data are combined from three experiments. *, $P < 0.05$ for ERα S522A versus wt ERα or other S-to-A mutant receptors. (Right) Protein blot demonstrating the purity of the membrane preparation. Caveolin 1 (Cav1) and 5′ nucleotidase (5′NT) are integral membrane proteins, while transportin and NTF-2 are nuclear proteins. β-COP is a Golgi protein.

lin 1 was clearly visualized at the membrane (Fig. 1E, panels B and E). Colocalization of membrane wt ERα with caveolin 1 (Fig. 1E, panel C, arrow) was also seen for the S522A receptor (panel F), but the latter showed decreased amounts colocalized, reflecting a decreased number of receptors at the membrane.

We next compared the binding of wt or S522A mouse ERα to that of S10A and S582A ERα constructs expressed in CHO cells (Fig. 1F). Total specific binding of E2 was determined in the nucleus and PM and revealed that both of the two additional mutant receptors were very similar to the wt receptor in both compartments. By comparison, S522A expression again exhibited significantly lower binding at the membrane only. These data indicate the specificity of S522 for ER localization at the cell surface.

Dissection of the contribution of other domains of ERα to membrane localization and function. It is possible that elements contained within other domains of ERα contribute importantly to membrane localization. In this respect, Schlegel et al. (37) have recently shown that residues 1 to 282 (the A/B and C domains) of human ERα bind to caveolin 1, a largely membrane localized protein that facilitates membrane localization of ER (31) and that, when overexpressed, promotes nuclear ER localization and transcriptional action. We therefore asked whether mutant ERα that lacked either the A/B or the C domain was capable of localizing to the PM and signaling to ERK. We compared the effects of CHO cells expressing these deletion or truncation mutants to those of CHO cells expressing wt ERα. We found that HE11G (with the A/B domain deleted) and H19G (with the C domain deleted) were comparable to wt ERα, both in specific binding of E2 at the cell membrane and in ERK activation by the steroid (Fig. 2). In contrast, a mutant with helix 12 and the F domain deleted,

TABLE 1. Binding characteristics of wt or S522A mutant mouse ERα expressed in the nuclei and membranes of CHO-K1 cells

Localization	K_d (nM)[a]	B_{max} (pmol/mg of protein)[a]
Nuclear		
wt ERα	0.26 ± 0.02	422 ± 38*
Mutant ERα	0.25 ± 0.02	395 ± 26*
Plasma membrane		
wt ERα	0.28 ± 0.04	17.2 ± 3.8†
Mutant ERα	0.27 ± 0.03	6.6 ± 0.26

[a] Data are from Scatchard analysis of competitive binding studies and are means ± standard errors of the means for three separate experiments combined. *, $P < 0.05$ for B_{max} of corresponding nuclear versus membrane ERα. †, $P < 0.05$ for B_{max} of wt ERα versus S522A mutant ERα.

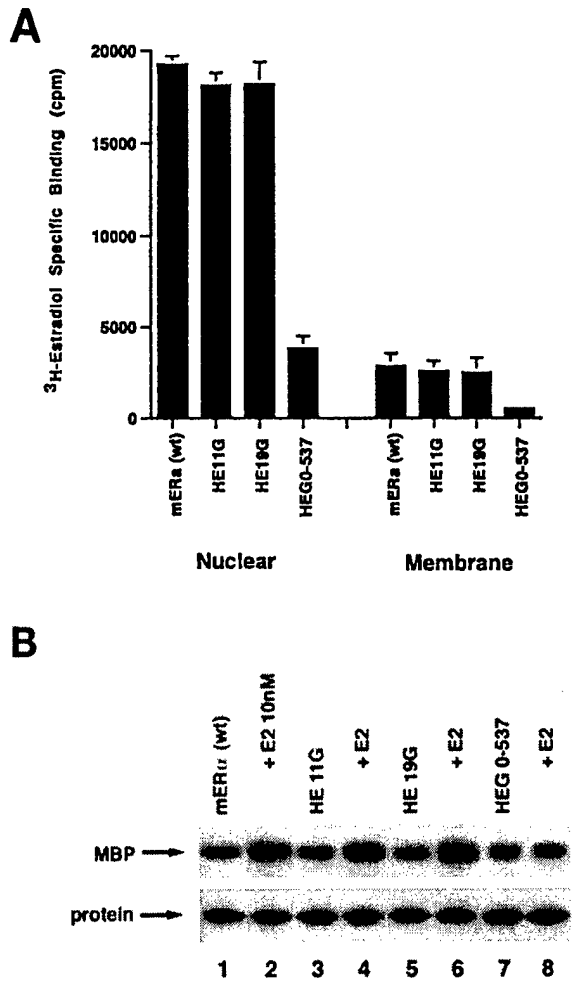


FIG. 2. (A) Binding of E2 to the nuclei and membranes of CHO cells expressing either HE11G (A/B domain deleted), HE19G (C domain deleted), or HEG0-537 (helix 12 and F domain deleted) ERα or wt ERα. The study was repeated. (B) ERK activation in response to E2 in CHO cells expressing either wt ERα or a deletion mutant. Activity was determined after 8 min of incubation with 10 nM E2. MBP was used as a substrate for ERK activity. Total ERK protein is shown on the immunoblot beneath the activity results.

HEG0-537 (truncated at residue 537), specifically bound little E2 in either the nuclear or the membrane compartment and did not support E2 activation of ERK. Thus, the A/B and C domains do not contribute to membrane ER localization and signaling by E2. However, deleting a small but important region within the E domain (in conjunction with loss of the F domain) has a profound effect on E2 binding to any ER pool, as well as on membrane function. Further understanding of the specific residues within the E domain that are required for ER localization at the membrane will require the characterization of a very extensive series of conservative mutations within this region. These results support a focus on the E domain for further understanding of the compartmentalization of ER.

ERα S522A is less capable than wt ERα of signaling from the membrane. It was important to ascertain whether the loss of membrane receptors resulting from expression of the S522A mutant also affected signal transduction. We therefore compared ERK (MAP kinase) activation by E2 in CHO cells expressing wt or mutant ERα. E2 significantly stimulated ERK activity after 8 min of exposure to CHO cells expressing wt ERα (Fig. 3A, left). However, ERK activation in response to E2 was reduced by 68% in CHO cells expressing ERα S522A (relative to activation in cells expressing wt ERα) (Fig. 3A, left; compare lanes 2 and 4). Activation of ERK by E2 was further compared in CHO cells that expressed wt or S10A or S582A mutant receptors. Consistent with the binding data, the additional serine mutants were nearly identical to the wt in activation of this MAP kinase (Fig. 3A, right).

We then examined cAMP generation, reflecting adenylate cyclase activation in the membrane, and found that E2 was 57% less capable of generating this cyclic nucleotide in S522A mutant-expressing than in wt ERα-expressing CHO cells (Fig. 3B). Generation of cAMP often arises from Gαs stimulation, which was previously demonstrated in response to membrane ER activation by E2 (32). Finally, we measured IP generation (Fig. 3C) and found a significant (53%) difference in production between cells expressing the two types of ERα. IP generation commonly results from the activation of Gαq, which was previously shown to be stimulated by E2 activation of membrane ER expressed in CHO cells (32). These data indicate that the reduction in membrane ER levels seen with S522A protein expression has significant functional consequences, and they further support the idea that E2 activates signal transduction through the membrane (and not the nuclear) receptor. To further support the latter concept, we subcloned the full-length wt ERα into vectors that contain membrane or nuclear localization signals and also express a GFP fusion protein (ECFP; Clontech). We then expressed in CHO cells either nontargeted wt ERα or wt ERα targeted either to the membrane or to the nucleus. As seen in Fig. 3D, expression of the nontargeted wt ERα and especially the membrane-targeted receptor supported rapid ERK activation by E2. In contrast, there was no activation of ERK in CHO cells expressing nucleus-targeted ER. Combined with previous experiments targeting the E domain to the membrane or nucleus (31), these data show that it is the membrane ER that supports rapid kinase activation in response to E2.

Palmitoylation, myristylation, and glucosylphosphoinositol (GPI) anchor studies addressing ER localization at the membrane. A number of posttranslational (or cotranslational) processes have been found to facilitate the movement and anchoring of proteins in the PM. To determine whether any of these alterations helped explain how ER localized to the membrane, we examined lipid modifications of ER. We expressed the wt mouse ERα in CHO cells and then labeled the cells with [^{3}H]palmitate or myristic acid. As expected, there was no uptake or incorporation of either lipid into the nuclear ERα. Furthermore, we found that there was no incorporation of myristate into the membrane ERα, consistent with the lack of a consensus myristylation site determined either from our partial sequence of membrane ERα or from viewing the known full-length sequence (48). Similarly, a possible but nonclassic palmitoylation site was identified from a peptide corresponding to a region in the mouse ERα C terminus, encompassing serine 522. However, in the membrane, there was no specific incorporation of palmitate into ERα in either the presence or the absence of E2 (data not shown). Other modifications, such

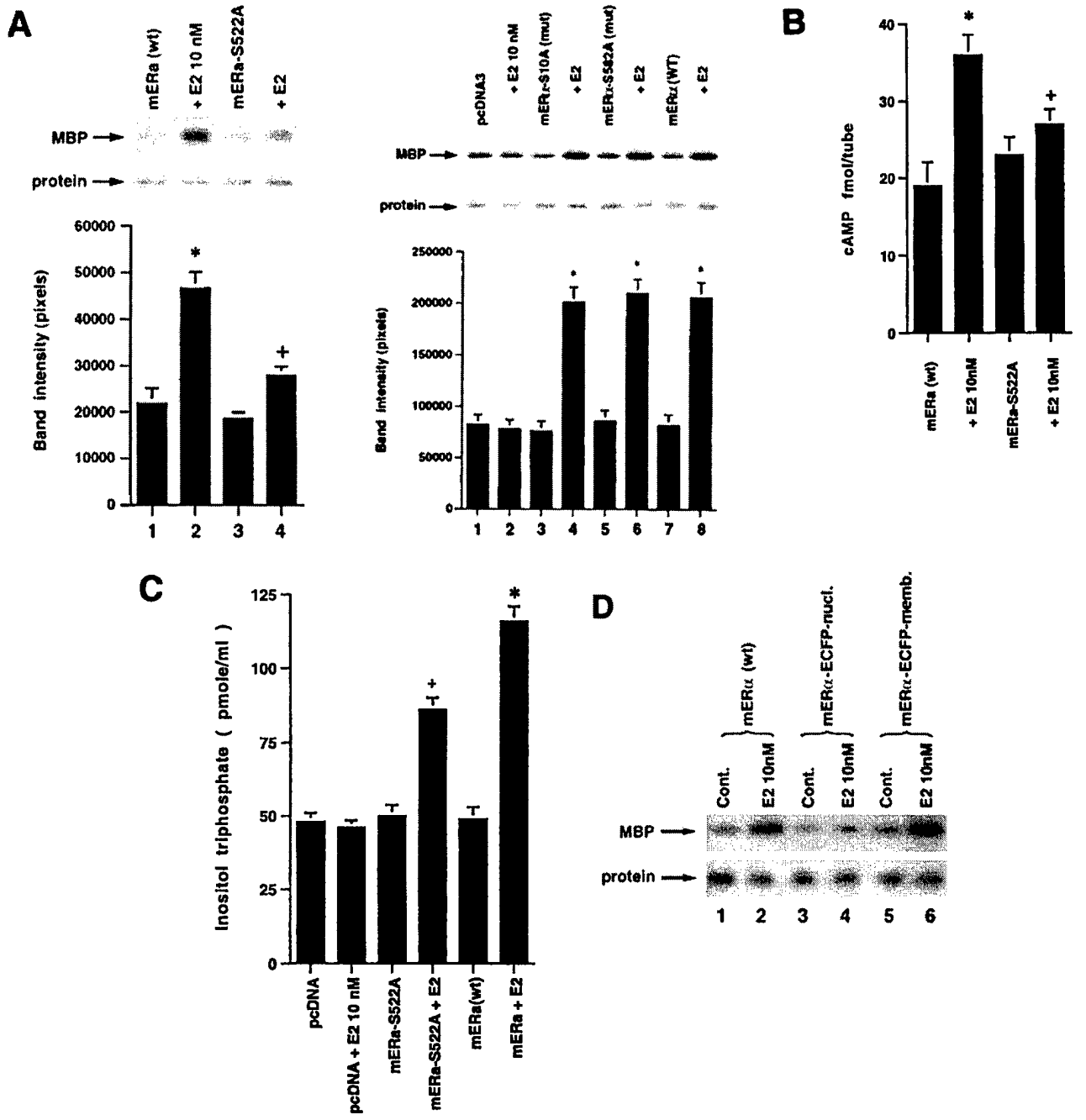


FIG. 3. (A) (Left) ERK activity is stimulated by E2 in CHO cells expressing wt ERα but less so in CHO cells expressing S522A mutant ERα. *, $P < 0.05$ for E2 versus the control (mouse ERα [mERα] without E2) in three combined experiments; +, $P < 0.05$ for ERK response to E2 in CHO cells expressing wt ERα (lane 2) versus ERα S522A (lane 4) in three combined experiments. (Right) Comparable stimulatory effects by E2 on ERK activity in CHO cells expressing wt ERα or the S10A or S582A mutant (lanes 3 to 8). Lanes 1 and 2 show that the intrinsic ERK activity of CHO cells expressing the empty vector, pcDNA3, cannot be stimulated by E2, due to a lack of endogenous ER. *, $P < 0.05$ for control versus E2. (B) Generation of cAMP in response to E2 in CHO cells expressing wt or S522A mutant ER. (C) IP3 generation in response to E2 in the above cells. Bar graph data are means ± standard errors of the means from triplicate determinations per experiment and are from two (cAMP) or three (IP3) combined studies. (D) Targeting ER to the membrane but not the nucleus allows E2 to rapidly activate ERK. CHO cells were transfected to express either nontargeted wt mERα or wt mERα targeted to the nucleus (mERα-ECFP-nucl.) or the membrane (mERα-ECFP-memb.). Cont., control. The study was repeated.

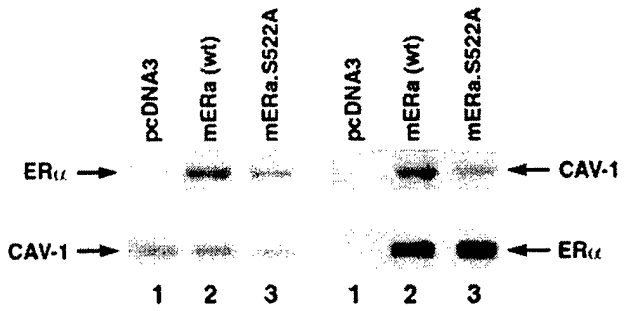


FIG. 4. ERα and caveolin 1 (CAV-1) association in the cytoplasm. CHO-K1 cell cultures (100-mm-diameter dishes) were transfected with 10 μg of wt or S522A mutant ERα plasmid DNA. The cells were lysed, and immunoprecipitation for caveolin 1 was carried out, followed by immunoblotting for ERα (left panels); or the order was reversed (right panels). Results shown are representative of three experiments. Caveolin 1 and ERα immunoblots are shown (lower panels) to demonstrate equal gel protein loading and equal expression of the two ER. mERα, mouse ERα.

as farnesylation or geranylgeranylation, usually require concomitant palmitoylation and occur at the very end (usually the N terminus) of the protein. No such sites were identified. Thus, we conclude that ERα is probably not posttranslationally lipidated to effect membrane translocation.

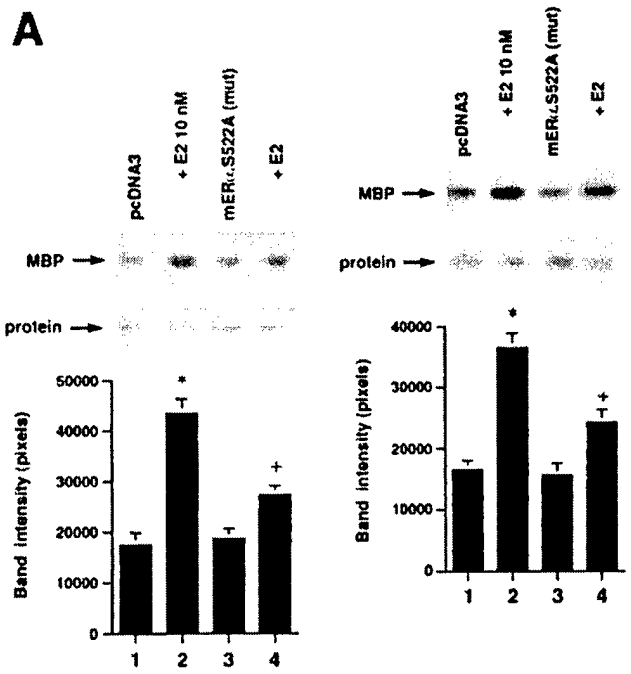
Addition of GPI to a protein in the Golgi complex serves to anchor such modified proteins in the extracellular leaflet of the PM (21). The PI-PLC enzyme cleaves GPI-modified proteins and therefore releases these membrane proteins into the culture medium, so that they cannot be detected by binding ligand at the cell surface. We found that treatment of the ER-expressing CHO cells with this phospholipase did not change the binding of E2 to ER in the PM. Furthermore, the anchoring of ER in the outer leaflet of the PM would generally preclude its localization in the caveolae, a membrane domain where ER has now been detected (6, 13). Thus, it is unlikely that ER undergoes this posttranslational (cotranslational) modification.

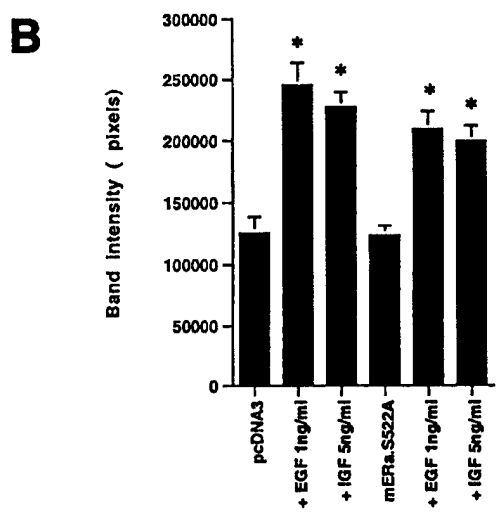
Interactions of wt or S522A mutant ERα with caveolin 1. It was recently reported that endogenous ER physically associates with the caveolin 1 protein in both the PM and cytosol of endothelial, vascular smooth muscle, and MCF-7 cells (31). Furthermore, expression of full-length caveolin 1 in MCF-7 or Caco-2 cells facilitates the movement of ER from the cytosol to the PM. Thus, ER-caveolin binding is important for the ability of ER to localize to the PM. We therefore examined whether the S522A mutant receptor bound to caveolin 1 comparably to wt ERα. This was accomplished in CHO cells, where we expressed the two ER constructs and utilized the endogenous caveolin in these cells. The ER was immunoprecipitated from the CHO cells, followed by immunoblotting for caveolin 1 (and vice versa), and association was examined in the absence of E2. In the cytosol of CHO cells, expression of either wt or S522A mutant ERα resulted in the receptor complexing with endogenous caveolin 1. However, the association of ERα S522A with caveolin 1 was 60% lower than that of wt ERα (Fig. 4). Importantly, as shown, the total ER levels expressed from the two vectors were comparable. These data are compatible with the idea that S522 is important for binding to caveolin 1, a protein that facilitates the membrane localization of the steroid binding protein (31). Thus, we have identified a mechanism to explain why ERα S522A is poorly localized to the PM.

Expression of S522A inhibits endogenous membrane ER function. We then asked whether the expression of ERα S522A interferes with the function of endogenous E2-ER signaling from the membrane. To test this hypothesis, we transiently expressed ERα S522A in MCF-7 and ZR-75-1 breast cancer cells. It was previously shown that E2 induced rapid signaling from membrane ER in these cells (33). In MCF-7 cells transfected to express pcDNA3 (control), E2 caused a twofold activation of ERK activity via the endogenous membrane ER (Fig. 5A, left; compare lane 1 with lane 2). In contrast, ERα S522A-expressing cells responded to E2 with 61% less activation of ERK (Fig. 5A, left; compare lanes 1 and 2 with lanes 3 and 4). Comparably, ERα S522A expression resulted in a 70% decrease in ERK activation in E2-treated ZR-75-1 cells (Fig. 5A, right; compare lanes 1 and 2 with lanes 3 and 4). We also determined that activation of ERK by epidermal growth factor (EGF) or IGF-1 in MCF-7 cells (Fig. 5B, first three columns) was not significantly affected by expression of the mutant ER (last three columns). This demonstrates the specific action of ERα S522A to impair only E2-ER signaling, and it also indicates that signaling by the two growth factors does not require an intact membrane ER signaling system. To further establish the specificity of these results, we expressed the S10A mutant in MCF-7 cells. There was no difference in ERK activation in response to E2 between cells expressing only endogenous ER (pcDNA3 transfected) and the same cells additionally transfected with ERα S10A (data not shown).

The inability of ERα S522A to fully localize to the membrane contrasted with the normal amount and function of nuclear ER when this construct was expressed in CHO cells. We therefore determined the specificity of S522A to serve as a dominant-negative protein in MCF-7 cells for membrane but not nuclear ER. MCF-7 cells were transiently transfected with an ERE-luciferase reporter in the presence or absence of coexpression of ERα S522A or pcDNA3. In pcDNA3-expressing cells, E2 caused a dose-related, 2.5-fold maximal stimulation of luciferase function (Fig. 5C). When the S522A mutant was expressed in these cells, transactivation of this reporter by E2 was comparable. This indicates that expression of the mutant ERα did not affect endogenous nuclear ER function.

To understand the cell biological effects of S522A expression and the role of the endogenous membrane ERα, we examined the ability of estrogen to promote cyclin D1 protein expression and cdk4 activation in MCF-7 cells. It has previously been shown that, in response to growth factors, signaling through ERK to Ets protein phosphorylation transactivates the cyclin D1 promoter and stimulates cyclin D1 protein synthesis (1, 15). E2 has been shown to stimulate cyclin D1 transcription and protein synthesis (47). We found that E2 was capable of increasing cyclin D1 protein levels nearly threefold (Fig. 6A). This was significantly related to ERK activation, since the MEK inhibitor PD98059 substantially prevented this effect. It was previously shown that PD98059 completely blocked E2 activation of ERK in MCF-7 cells (34). Importantly, expression of ERα S522A inhibited the E2-induced increase in cyclin D1 protein levels by 68%. Thus, the ability of ERα S522A to inhibit ERK activation arising from the endogenous mem-





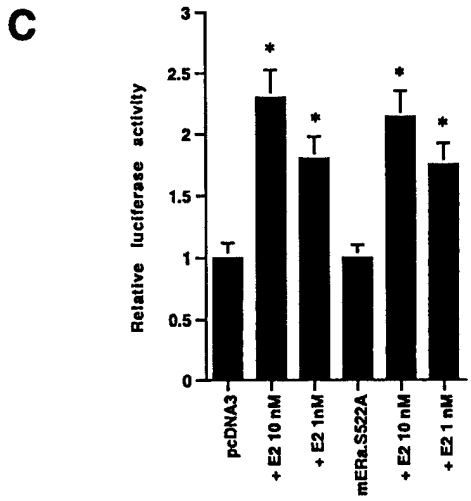


FIG. 5. (A) Expression of ERα S522A inhibits E2 activation of ERK. MCF-7 (left) or ZR-75-1 (right) breast cancer cells (which express endogenous ER) were transfected transiently to express either pcDNA3 (control) or S522A mutant ERα. The cells were then exposed to 10 nM E2 for 8 min, after which they were lysed, and immunoprecipitated ERK was assayed for activity by using MBP as a substrate. Precipitated ERK protein is shown in the lower gels, and the bar graphs each reflect three experiments combined. *, $P < 0.05$ for pcDNA3 in the absence versus the presence of E2; +, $P < 0.05$ for comparison of E2 treatments of pcDNA3-expressing versus ERα S522A-expressing cells. (B) ERα S522A does not impair EGF or IGF-1 activation of ERK. Data from three experiments are combined. *, $P < 0.05$ for pcDNA3-transfected or ERα S522A-expressing MCF-7 cells in the absence versus the presence of EGF or IGF-1. (C) E2 comparably activates an ERE-luciferase reporter in untransfected MCF-7 cells and MCF-7 cells transfected to express ERα S522A. Bar graph shows results for three experiments combined. *, $P < 0.05$ for pcDNA3- or ERα S522A-transfected MCF-7 cells without versus with E2.

brane ER greatly contributed to the inhibition of the increase in cyclin D1 protein levels.

We then determined the effect of E2 signaling on cdk4 activity. We found that E2 stimulated the important phosphorylation of the retinoblastoma (Rb) protein by this kinase 2.5-fold (Fig. 6B). Inactivation of Rb results from its phosphorylation mainly by cyclin D1-cdk4 and possibly by cyclin E-cdk2 and allows G_1/S progression in many cell types (39). Expression of S522A resulted in a 70% decrease in the ability of E2 to activate cdk4 activity. The results indicate that signaling from the membrane ER is important for a G_1 event that is essential to breast cancer cell cycle progression.

We also assessed G_1/S progression, determined by thymidine incorporation into DNA. It has previously been shown in MCF-7 or ER-expressing CHO cells that E2 stimulation of DNA synthesis is partly regulated through the ERK signaling pathway (5, 32). We found here that E2 caused a 70% increase in thymidine incorporation into DNA, a marker of the S phase. Two-thirds of this increase was blocked by PD98059. Thus, E2 utilizes several mechanisms to stimulate G_1/S progression, but ERK activation is the most important. Upon expression of ERα S522A, E2-induced thymidine incorporation was significantly reduced, by 50%. Thus, expression of the mutant ERα affirms the participation of the membrane steroid receptor in this event.

It would be important to know if the dominant-negative effect of the S522A mutant ERα extended to other cells. It was previously shown that E2 activates p38β MAP kinase in endothelial cells through endogenous membrane ER and that this leads to the angiogenic and cell survival effects of E2 in these

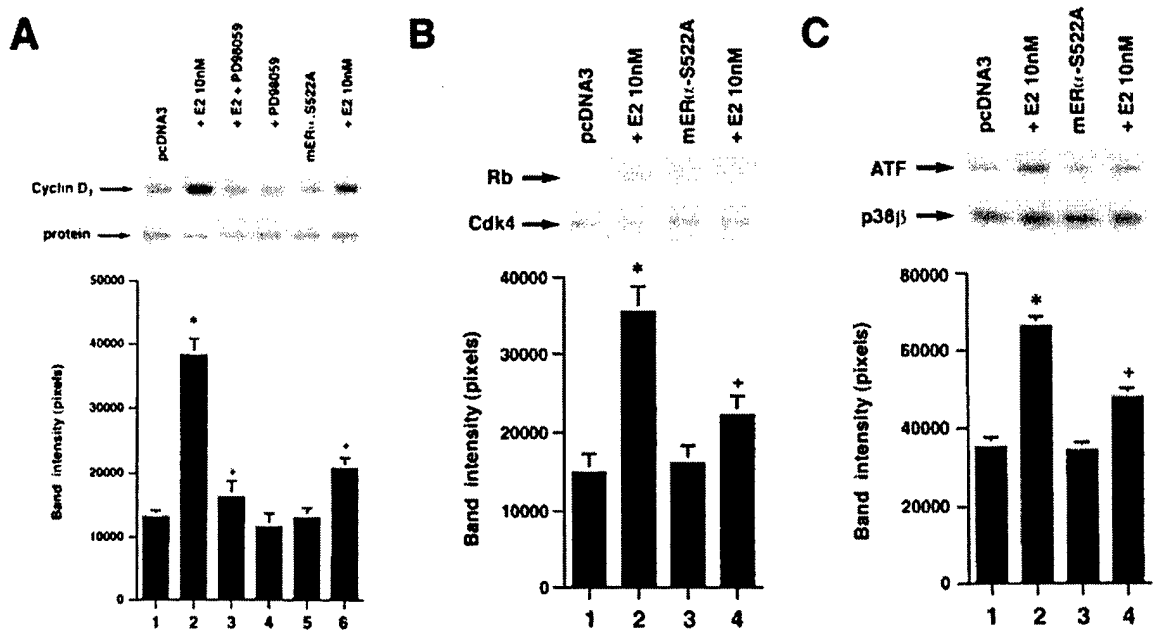


FIG. 6. (A) Cyclin D1 expression is increased in response to E2 and is dependent on ERK activation in wt MCF-7 cells. MCF-7 cells transfected to express ERα S522A show a lower response to 100 nM E2. Data are representative of three experiments, which were combined for the bar graph. *, $P < 0.05$ for pcDNA3-expressing cells without versus with E2; +, $P < 0.05$ for cells incubated with pcDNA3 plus E2 versus the same condition plus 10 μM PD 98059, or versus cells cotransfected with ERα S522A. (B) cdk4 activity is significantly downregulated by ERα S522A in MCF-7 cells. Cells were transfected with pcDNA3 or the mutant ER and exposed to 10 nM E2 for 6 h. cdk4 kinase was immunoprecipitated, and an in vitro assay of activity was accomplished by using Rb protein as a substrate. Bar graph data are from three experiments combined. (C) E2-stimulation of p38β activity in endothelial cells is inhibited by ERα S522A. Transfected endothelial cells were incubated with E2 for 20 min, and p38β activity was determined against the substrate protein ATF-1. Data are from three experiments.

cells (33). Here, we report that E2 activation of p38β in endothelial cells is 60% reduced when ERα S522A is expressed (Fig. 6C). Thus, this mutant ER may be a useful tool for determining the contributions of the membrane ERα to various cell-signaling and biological functions.

Mechanisms of ERα S522A inhibition of endogenous ER function. How might ERα S522A inhibit endogenous ER function? One possibility to explain the dominant-negative effect of ERα S522A is that it might heterodimerize with wt ER. Dimerization is necessary for ER to transactivate genes (43), and heterodimerization between ERα and ERβ has been reported to inhibit ERα function (29). Since the S522A mutant does not translocate effectively to the PM, it could potentially bind and sequester the endogenous receptor, thus interfering with its signaling function. To assess possible heterodimerization, we expressed GFP-tagged ERα S522A and His-tagged wt ERα in CHO cells and performed pulldown studies. After lysis, the cell extracts were immunoprecipitated and blotted with anti-GFP and anti-His antibodies, in both orders. After E2 treatment of cells where either or both tagged forms of the receptor were expressed, we found evidence for homodimerization and heterodimerization of wt and mutant ERα (Fig. 7A). As specificity controls, the fourth lanes show a lack of ER when His-tagged wt ERα is expressed and immunoprecipitated but blotting is done with an antibody to GFP (Fig. 7A, left), and when GFP-tagged ERα S522A is expressed but blotting is done with an antibody to His (Fig. 7A, right). Furthermore, S522A mutant ERα was as capable as wt ERα of dimerizing to wt ERα (Fig. 7A, left). These data indicate that ERα S522A can bind to wt ERα, thereby potentially sequestering or otherwise limiting endogenous receptor signaling from the membrane.

To further examine this mechanism, we determined the membrane localization of wt ERα in CHO cells transfected to express equal amounts of either (i) GFP-tagged wt ERα plus His-tagged wt ERα or (ii) GFP-tagged wt ERα plus His-tagged ERα S522A. As seen in Fig. 7B, expression of ERα S522A substantially decreased the membrane localization of GFP-tagged wt ERα. By contrast, nuclear receptor expression was not different. Thus, ERα S522A inefficiently translocates to the cell surface and also prevents wt ERα PM localization after heterodimerization. These results provide a mechanism for the dominant-negative action of the mutant ERα, but other effects are tenable.

Membrane wt ERα and S522A mutant ERα bind equally to signaling molecules. It is also important to consider that mutation of serine 522 to alanine might disturb the inherent ability of membrane ER to associate with important signaling molecules. This could contribute to the differential signaling from the membrane by wt versus S522A mutant ERα. To investigate this, we transfected CHO cells to express either wt or S522A mutant ERα. We immunoprecipitated the ER from membrane preparations and then normalized the proteins for equivalent amounts of receptor(s), as indicated by Western blotting (Fig. 7C). We then took separate (normalized) aliquots of immunoprecipitated wt ERα or ERα S522A and immunoblotted the aliquots for Ras or Raf. We found that the two receptors associated equally with the Ras or Raf signaling molecules in the presence of the steroid (Fig. 7C, left). Control

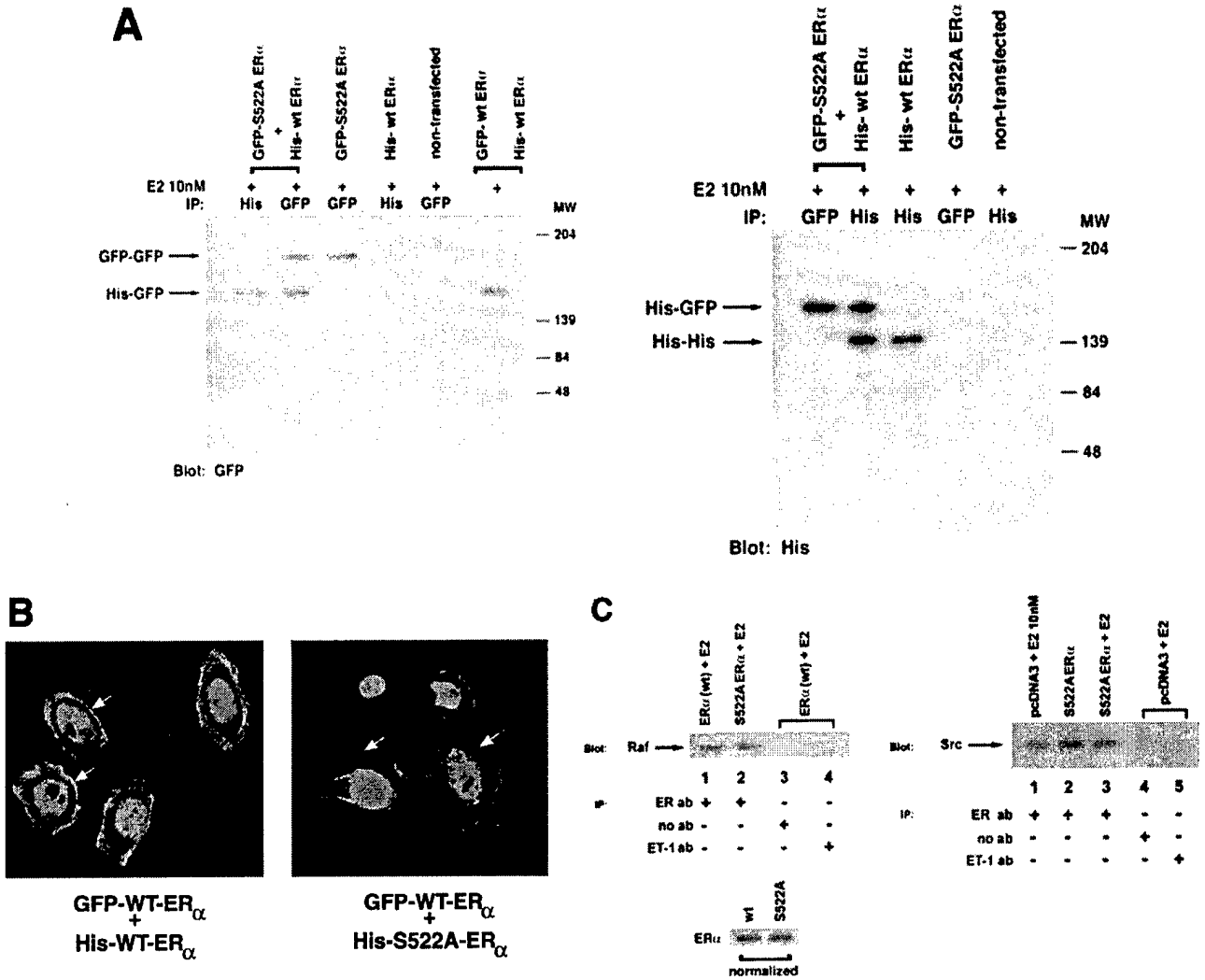


FIG. 7. (A) Homo- and heterodimerization of wt and ERα S522A after expression in CHO cells. Doubly or singly transfected cells were first exposed to 10 nM E2 for 10 min and then lysed, and the lysate underwent immunoprecipitation (IP) with an antibody to His or GFP, as indicated, eventually followed by blotting with an antibody to GFP (left) or to His (right). Proteins were separated by SDS-PAGE under nonreducing conditions (native gel). Molecular weight markers indicate the different sizes of the GFP-tagged wt ERα and His-tagged ERα S522A homodimers and the intermediate size of the heterodimer. (B) Expression of S522A prevents wt ERα localization at the membrane. CHO cells were transfected with equal amounts of plasmids encoding GFP-tagged wt ERα plus His-tagged wt ERα (10 μg of total DNA/100-mm-diameter dish) or with GFP-tagged wt ERα plus His-tagged ERα S522A. Localization of receptors was determined by confocal microscopy. (C) wt and S522A mutant ERα equally associate with Ras, Raf, or Src at the membrane. (Left) CHO cells were transfected to express either receptor, and after normalization, equal receptor protein aliquots were confirmed by Western blotting. Equal protein aliquots were used for immunoblots to determine wt or mutant ERα association with Ras or Raf. The study was carried out in the presence of 10 nM E2 for 10 min and was repeated. Immunoprecipitation with no antibody (ab) or an irrelevant antibody to the endothelin-1 peptide did not yield a protein band. (Right) MCF-7 cells were transfected with His-tagged ERα S522A (lanes 2 and 3) or with His-tagged pcDNA3 (lane 1) and were incubated or not with 10 nM E2 for 10 min. The cells were lysed and immunoprecipitated for ERα (lane 1) or for His (lanes 2 and 3), then immunoblotted for Src. Expression of His-tagged pcDNA3 did not coprecipitate Src (data not shown).

immunoprecipitations without an antibody or with an irrelevant antibody to endothelin-1 (ET-1) did not bring down a Ras or Raf protein band. Especially important is the association of ER with Src, which has been reported to bind to tyrosine 537 of ERα (24). We examined the effect of expression of ERα S522A on subsequent Src association with ER in MCF-7 cells. As seen in Fig. 7C (right), endogenous ERα associated with Src comparably to the S522A mutant receptor, and this was unaffected by E2. This suggests that there is no alteration of association with important signaling molecules by ERα S522A that can account for the differences in signaling from the membrane. Thus, we believe that it is primarily the membrane receptor number that determines the differences in signaling.

DISCUSSION

The presence of a PM ER in human cells that enacts signal transduction and thereby contributes to the cellular effects of

E2 is increasingly accepted (14, 33, 34, 40, 49). E2 signaling through PI 3-kinase in vivo rescues muscle from ischemia-reperfusion injury (40), while activation of ERK prevents breast cancer (34) or osteoblast (14) cell death. Recently, Marquez and Pietras have shown that administration of antibodies to ERα in nude mice blocked the growth of human breast cancer xenografts (23). This probably resulted from inhibition by the antibodies of membrane ER signaling to ERK and PI 3-kinase. Spatially, the receptor appears to be localized primarily but not exclusively to caveolar fractions of the PM (6, 13). In this confined area, ER potentially interact with a variety of signaling molecules that must localize to the PM for activation. The PM ER acts as a G protein-coupled receptor, directly (31, 50) or indirectly (10), leading to activation of multiple signaling pathways. This results in cAMP generation (4), PLC and inositol triphosphate (IP3) activation (16, 41), and the stimulation of cascades leading to enhanced activity of ERK, JNK, and p38 MAP kinases (23, 32, 33). The importance of these nongenomic mechanisms of estrogen action is analogous to that of the actions of steroids in plants. In *Arabidopsis* spp., brassinosteroids mediate plant cell developmental growth and fertility (22), and cell action results from steroid binding to a transmembrane, tyrosine kinase receptor protein (44). Thus, steroid action at the cell surface is an ancient function conserved from plants to humans, further indicating its importance.

One important issue with regard to the cell surface ER that we addressed here is the structural requirements for a population of ER to translocate to the PM. We found that ER are not posttranslationally lipidated, as occurs with other PM-localized proteins. Rather, we identified serine 522 as important for membrane translocation. Compared to expression of wt mouse ERα, mutation of this serine to alanine resulted in 62% fewer receptors expressed at the membrane, with little influence on receptor affinity for ligand. However, there was no appreciable effect on the nuclear receptor numbers, affinity, and function (transactivation of an ERE-luciferase reporter). Furthermore, expression of the S522A mutant receptor was markedly less efficient in supporting E2-induced ERK activation, cAMP generation, and stimulation of IP3 than wt receptor expression. Presumably, reduced signaling resulted from a decreased number of receptors available at the membrane. Supporting this, we did not find a loss of association at the membrane between S522A mutant ERα and signaling molecules, compared to that for wt ERα. However, this receptor can serve as a dominant-negative protein for wt ER when expressed in MCF-7 cells, and therefore additional mechanisms of abolishing signal transduction may be relevant (see below). Supporting the specificity of our results, we found that substitution of alanine for serine at residues 10 and 582 of the mouse ERα had no effect on either E2 binding to the membrane or signaling by E2, when these mutants were compared to wt ERα.

How does ERα localize to the membrane, and how does S522 contribute? It was recently determined that caveolin 1 protein facilitates the translocation of ER to the PM and that the two endogenous proteins physically bind in both the cytosol and the PM (31). The scaffolding domain of caveolin 1 (amino acids 82 to 101) is essential for this protein to move from the cytosol to the membrane (3, 27), and we determined that the scaffolding domain facilitates ER movement to the PM. An important question, then, is whether serine 522 is necessary for the association of ERα and caveolin 1. We report here that in the cytoplasm, the physical association between these two proteins was 60% decreased by the mutation of serine 522. In contrast, association of caveolin with S10- or S582-mutated ERα was comparable to that with wt ER (data not shown). It has recently been shown that residues 1 to 282 of ERα bind to caveolin 1 (37). However, Lu et al. recently showed that caveolin 1 associates with the androgen receptor through both N-terminal (A/B domain) and E domain elements (20). We found that an A/B domain deletion mutant ERα localizes to the membrane and supports E2 signaling to ERK equivalently to wt ERα. Thus, the interaction between caveolin and the N terminus of ER may not be functionally important for the membrane ER.

What supports the relevance of S522A for ER action at the membrane? Kousteni et al. showed that by targeting only the E domain of ERα to the PM but not to the nucleus, E2 could rescue cells from apoptotic death (14). It was recently demonstrated that the E domain (and here the full-length ER) is sufficient to convey robust ERK activation in response to E2 when targeted to the PM (31). These overall findings suggest that the E domain is generally important for ERα actions originating at the membrane. Identification of serine 522 provides a novel insight into the specific structural requirements for membrane localization, steroid action, and cell biological functions of E2. We suggest that similar examination of the role of the ligand binding domains of the progesterone, androgen, and other steroid receptors is warranted.

To establish the roles of the membrane ER in cell biology, several approaches could be taken. Targeting of ER to only one compartment in the cell may suggest a specific function for a pool of the endogenous receptor. Another approach is to devise specific agonists or antagonists for the membrane ER, reagents that do not enter the cell to bind the nuclear receptor. Several ER agonists have recently been described that dissociate some membrane signaling from transcriptional activity (14). However, ER signaling through the membrane receptor stimulates gene transcription (9, 46), and thus, these two functions may not always reflect membrane versus nuclear receptor action. A third approach is to express mutant sex steroid receptors that specifically interfere with endogenous ER actions at the membrane. We show here in MCF-7, ZR-75-1, and endothelial cells that ERα S522A is capable of significantly preventing E2 signaling from the endogenous membrane receptor. We propose that this could result from preventing endogenous ER localization at the membrane. Since the dimerization motifs for ERα do not involve serine 522, we reasoned that wt and mutant ER could heterodimerize and thus sequester wt ER from localizing fully at the PM. Supporting this, we provide evidence of heterodimerization between the mutant and wt ERα and a loss of membrane wt ERα when both receptors are coexpressed.

In MCF-7 or ZR-75-1 breast cancer cells, expression of ERα S522A interfered with endogenous ER function. Expression of ERα S522A inhibited E2-induced ERK activation, cyclin D1 production, cdk4 activity, and G_1/S progression. Many of these actions of E2 require signaling from the membrane to kinases such as ERK. Furthermore, the utility of this approach was

shown in a second cell type, where membrane E2-ER signaling to p38β MAP kinase (33) was significantly prevented by expression of ERα S522A. The strong inhibition of cyclin D1 protein in MCF-7 cells by ERα S522A expression and the linkage to modulation of ERK activity suggests an important therapeutic intervention in breast cancer. In vitro, E2 induction of cyclin D1 overcomes the tamoxifen-induced G_1/S cell cycle block (47). Also, tamoxifen sensitivity can be restored through p27 function, resulting from ERK downregulation (8). In this respect, limiting endogenous membrane ER signaling to ERK (19) and cyclin D1 may be therapeutically desirable, as suggested by our use of the S522A mutant ERα. It has also been recently reported that specifically cyclin D1 is essential to the development of rodent breast cancer, resulting from Ras or Neu oncogene signaling (51). Cyclin D1 has several important functions, but arguably the most important is the regulation of the inactivating phosphorylation of the Rb protein by cdk4, allowing E2F release and the subsequent transcription of genes that drive cell cycle progression in breast cancer (39). Our demonstration that ERα S522A significantly limits these events both points out therapeutic targets and reveals the importance of E2 signaling from the membrane. The ultimate goal of hormone replacement after the menopause is to activate specific, desirable effects of sex steroids (osteoblast survival) without invoking unwanted actions (breast cancer proliferation). This strategy will be best served by defining the array of discrete actions of E2 that result from binding at membrane and nuclear ER in various target cells. Expression of ERα S522A may be very useful in this regard.

ACKNOWLEDGMENTS

This work was supported by grants from the Research Service of the Department of Veterans Affairs, the Avon Products Breast Cancer Research Foundation, the Department of Defense Breast Cancer Research Program (grant BC990915), and the NIH (HL-59890) (to E.R.L.).

We thank M. Lisanti for caveolin plasmids.

REFERENCES

1. **Albanese, C., J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, and R. G. Pestell.** 1995. Transforming p21 ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem. **270:** 23589–23597.
2. **Alton, G., M. Hasilik, R. Niehues, K. Paneerselvam, J. R. Etchison, F. Fana, and H. H. Freeze.** 1998. Direct utilization of mannose for mammalian glycoprotein biosynthesis. Glycobiology **8:**285–295.
3. **Anderson, R. G.** 1998. The caveolae membrane system. Annu. Rev. Biochem. **67:**199–225.
4. **Aronica, S. M., W. L. Kraus, and B. S. Katzenellenbogen.** 1994. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc. Natl. Acad. Sci. USA **91:**8517–8521.
5. **Catoria, G., M. V. Barone, M. Di Domenico, A. Bilancio, D. Ametrano, A. Migliaccio, and F. Auricchio.** 1999. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. EMBO J. **18:**2500–2510.
6. **Chambliss, K. L., I. S. Yuhanna, C. Mineo, P. Liu, Z. German, T. S. Sherman, M. E. Mendelsohn, R. G. W. Anderson, and P. W. Shaul.** 2000. Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ. Res. **87:**e44–e52.
7. **Cooper, L. F., J. C. Tiffee, J. P. Griffin, H. Hamano, and Z. Guo.** 2000. Estrogen-induced resistance to osteoblast apoptosis is associated with increased hsp27 expression. J. Cell. Physiol. **185:**401–407.
8. **Donovan, J. C. H., A. Milic, and J. M. Slingerland.** 2001. Constitutive MEK/MAPK activation leads to p27kip1 deregulation and antiestrogen resistance in human breast cancer cells. J. Biol. Chem. **276:**40888–40895.
9. **Duan, R., W. Xie, and S. Safe.** 2001. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J. Biol. Chem. **276:**11590–11598.
10. **Filardo, E. J., J. A. Quinn, K. I. Bland, and R. A. Frackelton, Jr.** 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, gpr30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. **14:**1649–1660.
11. **Greene, G., N. Sobel, W. King, and E. Jensen.** 1984. Immunochemical studies of estrogen receptors. J. Steroid Biochem. **20:**51–56.
12. **Holt, A., G. Alton, C. H. Scaman, G. Loppnow, A. Szpacenko, I. Svendsen, and M. M. Palcic.** 1998. Identification of the quinone growth cofactor in mammalian semicarbazide-sensitive amine oxidase. Biochemistry **37:**4946–4957.
13. **Kim, H. P., J. Y. Lee, J. K. Jeong, S. W. Bae, H. K. Lee, and I. Jo.** 1999. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem. Biophys. Res. Commun. **263:**257–262.
14. **Kousteni, S., T. Bellido, L. I. Plotkin, C. A. O'Brien, D. L. Bodenner, L. Han, K. Han, G. B. DiGregorio, J. A. Katzenellenbogen, B. S. Katzenellenbogen, P. K. Roberson, R. S. Weinstein, R. L. Jilka, and S. C. Manolagas.** 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell **104:**719–730.
15. **Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur.** 1996. Cyclin D1 expression is regulated by the p42/p44 MAPK and negatively by the p38/HOG MAPK pathway. J. Biol. Chem. **271:**20608–20616.
16. **Le Mellay, V., B. Grosse, and M. Lieberherr.** 1997. Phospholipase C beta and membrane action of calcitriol and estradiol. J. Biol. Chem. **272:**11902–11907.
17. **Levin, E. R.** 1999. Cellular functions of the plasma membrane estrogen receptor. Trends Endocrinol. Metab. **10:**374–376.
18. **Lieberherr, M., B. Grosse, M. Kachkache, and S. Balsan.** 1993. Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional non-nuclear receptors. J. Bone Min. Res. **8:**1365–1376.
19. **Lobenhofer, E. K., G. Huper, J. D. Iglehart, and J. R. Marks.** 2000. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ. **11:**99–110.
20. **Lu, M. L., M. C. Schneider, Y. Zhang, and J. P. Richie.** 2001. Caveolin-1 interacts with androgen receptor: a positive modulator of androgen receptor mediated transactivation. J. Biol. Chem. **276:**13442–13451.
21. **Lublin, D.** 1992. Glucosyl-phosphatidylinositol anchoring of membrane proteins. Curr. Top. Microbiol. Immunol. **178:**142–168.
22. **Mandava, M. B.** 1988. Plant growth-promoting brassinosteroids. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39:**23–52.
23. **Marquez, D. C., and R. J. Pietras.** 2001. Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene **20:**5420–5430.
24. **Migliaccio, A., M. Di Domenico, G. Castoria, A. de Falco, P. Bontempo, E. Nola, and F. Auricchio.** 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. **15:**1292–1300.
25. **Morey, A. K., A. Pedram, M. Razandi, B. A. Prins, R.-M. Hu, E. Biesiada, and E. R. Levin.** 1997. Estrogen and progesterone inhibit human vascular smooth muscle proliferation. Endocrinology **138:**3330–3339.
26. **Norfleet, A. M., M. L. Thomas, B. Gametchu, and C. S. Watson.** 1999. Estrogen receptor-alpha detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. Endocrinology **140:**3805–3814.
27. **Okamoto, T., A. Schlegel, P. E. Scherer, and M. P. Lisanti.** 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J. Biol. Chem. **273:**5419–5422.
28. **Pedram, A., M. Razandi, and E. R. Levin.** 1998. Extracellular regulated kinase/jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. J. Biol. Chem. **273:**26722–26728.
29. **Pettersson, K., F. Delaunay, and J. A. Gustafsson.** 2000. Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene **19:**4970–4978.
30. **Pietras, R., and C. M. Szego.** 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature **265:**69–72.
31. **Razandi, M., P. Oh, A. Pedram, J. Schnitzer, and E. R. Levin.** 2002. Estrogen receptors associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol. Endocrinol. **16:**100–115.
32. **Razandi, M., A. Pedram, G. L. Greene, and E. R. Levin.** 1999. Cell membrane and nuclear estrogen receptors derive from a single transcript: studies of ERα and ERβ expressed in CHO cells. Mol. Endocrinol. **13:**307–319.
33. **Razandi, M., A. Pedram, and E. R. Levin.** 2000. Estrogen signals to preservation of endothelial cell form and function. J. Biol. Chem. **275:**38540–38546.
34. **Razandi, M., A. Pedram, and E. R. Levin.** 2000. Plasma membrane estrogen receptors signal to anti-apoptosis in breast cancer. Mol. Endocrinol. **14:** 1434–1447.
35. **Resh, M. D.** 1996. Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. Cell. Signal. **8:**403–412.
36. **Russell, K. S., M. P. Haynes, D. Sinha, E. Clerisme, and J. R. Bender.** 2000. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc. Natl. Acad. Sci. USA **97:**5930–5935.

37. **Schlegel, A., C. Wang, R. G. Pestell, and M. P. Lisanti.** 2001. Ligand-independent activation of oestrogen receptor α by caveolin-1. Biochem. J. **359**:203–210.
38. **Schnitzer, J. E., D. P. McIntosh, A. M. Dvorak, J. Liu, and P. Oh.** 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. Science **269**:1435–1439.
39. **Sherr, C. J.** 1993. Mammalian G_1 cyclins. Cell **73**:1059–1065.
40. **Simoncini, T., A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin, and J. K. Liao.** 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature **407**:538–541.
41. **Tesarik, J., and C. Mendoza.** 1995. Nongenomic effects of 17β-estradiol on maturing human oocytes: relationship to oocyte developmental potential. J. Clin. Endocrinol. Metab. **80**:1438–1443.
42. **Truss, M., and M. Beato.** 1993. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocrine Rev. **14**:459–479.
43. **Valentine, J. E., E. Kalkhoven, R. White, S. Hoare, and M. G. Parker.** 2000. Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transrepression. J. Biol. Chem. **275**:25322–25329.
44. **Wang, Z.-Y., H. Seto, S. Fujioka, S. Yoshida, and J. Chory.** 2001. BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature **410**:380–383.
45. **Watters, J. J., J. S. Campbell, M. J. Cunningham, E. G. Krebs, and D. M. Dorsa.** 1997. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology **138**:4030–4033.
46. **Watters, J. J., T. Y. Chun, Y. N. Kim, P. J. Bertics, and J. Gorski.** 2000. Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. Mol. Endocrinol. **14**:1872–1881.
47. **Watts, C. K., K. J. Sweeney, A. Warlters, E. A. Musgrove, and R. L. Sutherland.** 1994. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. Breast Canc. Res. Treat. **31**:95–105.
48. **White, R., J. A. Lees, M. Needham, J. Ham, and M. Parker.** 1987. Structural organization and expression of the mouse estrogen receptor. Mol. Endocrinol. **1**:735–744.
49. **Wise, P. M., D. B. Dubal, M. E. Wislon, S. W. Rau, and M. Bottner.** 2001. Neuroprotective effects of estrogen—new insights into mechanisms of action. Endocrinology **142**:969–973.
50. **Wyckoff, M. H., K. L. Chambliss, C. Mineo, I. S. Yuhanna, M. E. Mendelsohn, S. M. Mumby, and P. W. Shaul.** 2001. Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through $G\alpha_i$. J. Biol. Chem. **276**:27071–27076.
51. **Yu, Q., Y. Geng, and P. Sicinski.** 2001. Specific protection against breast cancers by cyclin D1 ablation. Nature **411**:1017–1021.

MOLECULAR AND CELLULAR BIOLOGY, July 2004, p. 001 APPENDIX 5 Vol. 24, No. 13
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.13.000–000.2004

BRCA1 Inhibits Membrane Estrogen and Growth Factor Receptor Signaling to Cell Proliferation in Breast Cancer

Mahnaz Razandi,[1,2] Ali Pedram,[1,2] Eliot M. Rosen,[3] and Ellis R. Levin[1,2,4]*

Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, California 90822;[1] Departments of Medicine[2] and Pharmacology,[4] University of California, Irvine, Irvine, California 92717; and Department of Radiation Oncology, Long Island Jewish Medical Center, New Hyde Park, New York 11040[3]

Received 28 October 2003 /Returned for modification 16 December 2003 /Accepted 6 April 2004

BRCA1 mutations and estrogen use are risk factors for the development of breast cancer. Recent work has identified estrogen receptors localized at the plasma membrane that signal to cell biology. We examined the impact of BRCA1 on membrane estrogen and growth factor receptor signaling to breast cancer cell proliferation. MCF-7 and ZR-75-1 cells showed a rapid and sustained activation of extracellular signal-related kinase (ERK) in response to estradiol (E2) that was substantially prevented by wild-type (wt) but not mutant BRCA1. The proliferation of MCF-7 cells induced by E2 was significantly inhibited by PD98059, a specific ERK inhibitor, or by dominant negative ERK2 expression and by expression of wt BRCA1 (but not mutant BRCA1). E2 induced the synthesis of cyclins D1 and B1, the activity of cyclin-dependent kinases Cdk4 and CDK1, and G_1/S and G_2/M cell cycle progression. The intact tumor suppressor inhibited all of these. wt BRCA1 also inhibited epidermal growth factor and insulin-like growth factor I-induced ERK and cell proliferation. The inhibition of ERK and cell proliferation by BRCA1 was prevented by phosphatase inhibitors and by interfering RNA knockdown of the ERK phosphatase, mitogen-activated kinase phosphatase 1. Our findings support a novel tumor suppressor function of BRCA1 that is relevant to breast cancer and identify a potential interactive risk factor for women with BRCA1 mutations.

AQ: A

Fn* Mutations of the BRCA1 and BRCA2 genes strongly increase the risk of developing breast and ovarian cancer in women and prostate cancer in men (2, 21, 46). The intact BRCA1 protein has several functions that prevent cancer development, including DNA repair, activation of cell cycle checkpoints, and induction of chromosome stability (10, 17, 22, 40, 46, 49). BRCA1 may induce p53-independent cell cycle arrest in breast cancer cells when highly overexpressed (11, 38) but more often collaborates with p53 for this function. The partnering of BRCA1 with the BARD protein activates ubiquitin E3 ligase activity and leads to proteasomal degradation of relevant proteins (1). These or other undefined functions that are compromised by AQ: B BRCA1 mutation contribute substantially to carcinogenesis.

Cells that transform in a background of BRCA1 mutation are initially hormone responsive. In one such malignancy, breast cancer, estradiol (E2) use after menopause is a risk factor (2, 35). Although many BRCA1 mutant-related breast tumors lack estrogen receptors (ER) at the time of diagnosis (16), some studies suggest that an interaction between estrogen and BRCA1 may contribute to early tumor pathogenesis. Bilateral prophylactic ovariectomy is associated with a significantly reduced incidence of breast cancer in women carrying mutant BRCA1 (33), while men with single allelic BRCA1 mutations have a much lower incidence of breast malignancy than women carrying the same mutation (42). Although E2 upregulates BRCA1 expression, this may indirectly reflect the proliferative actions of the sex steroid (19). It is unclear whether BRCA1 upregulation has biological consequences. More important, however, may be the functional interactions between the sex steroid and the wild-type (wt) tumor suppressor protein. BRCA1 inhibits both ligand-independent (54) and -dependent (6) transcription induced by nuclear ER. This inhibition results in part from the ability of the N terminus of BRCA1 (amino acids 1 to 300) to physically interact with the AF-2 domain of nuclear ERα (7). However, the impact of the interactions between BRCA1 and nuclear ER on breast cancer biology is unclear. AQ: C

E2 traditionally has been described to induce transcription through nuclear ER (48). However, E2 also rapidly activates both transcription and the modification of protein function through kinase activation (24, 29). Plasma membrane-associated ER usually mediates this signaling, activating G proteins and the extracellular signal-regulated protein kinase (ERK) cascade; this signal significantly influences the survival of the breast cancer patient (29). The majority of studies also suggest the possibility of a role for ERK in E2-induced cell proliferation (18, 30, 36). Breast cancer growth factors such as epidermal growth factor (EGF) or insulin-like growth factor I (IGF-I) utilize similar signaling pathways to stimulate cell proliferation, which results from the activation of their membrane tyrosine kinase receptors. Furthermore, membrane ER signaling to ERK in breast cancer results from cross talk to EGF AQ: D receptor (EGFR) transactivation (8). Thus, it is potentially AQ: E important to understand whether BRCA1 influences this mechanism of both membrane ER/E2 and growth factor action as a novel tumor suppressor function.

MATERIALS AND METHODS

Cell lines and materials. MCF-7, ZR-75-1, and HCC-1937 breast cancer cells were obtained from the American Type Culture Collection. Estradiol was obtained from Sigma, and ICI182780 was obtained from AstraZeneca (kindly

* Corresponding author. Mailing address: Medical Service (111-I), Long Beach VA Medical Center/UC—Irvine, 5901 E. 7th St., Long Beach, CA 90822. Phone: (562) 826-5748. Fax: (562) 826-5515. E-mail: ellis.levin@med.va.gov.

AUTHOR: Publication of this article cannot proceed without the signature of the person who read and corrected the proof on behalf of all the authors: _____ signature _____ date

provided by Alan Wakeling). EGF, IGF-I, tyrphostin AG1287, and PD98059 were purchased from Calbiochem. Antibodies were from Santa Cruz Biotechnology, Inc. Duplexed RNA oligomers for MKP-1, green fluorescent protein (GFP), and BRCA1 were synthesized by QIAGEN.

Kinase activity studies. For ERK activity assays, the cells were synchronized for 24 h in serum, phenol red, and growth factor-free medium. The cells were then exposed to 10 nM E2 for 9 min with or without additional substances, and the cells were then lysed and immunoprecipitated for ERK2 as previously described (25). Immunoprecipitated ERK2 samples were resuspended in 40 μl of kinase buffer containing [^{32}P]ATP and myelin basic protein (Sigma) as the substrate for the in vitro assay (25). Equal aliquots of immunoprecipitated ERK from each condition were also immunoblotted to show equal gel loading. All experiments were repeated two to three times.

For phosphatidylinositol 3-kinase (PI3K), phosphorylation of AKT at serine 473 was determined after 15 min of exposure to E2 as an indication of activation (25). Cultured cell lysates were pelleted and dissolved in sodium dodecyl sulfate sample buffer, boiled, separated, and then transferred to nitrocellulose. Phosphorylated AKT was detected by using phosphospecific monoclonal antibodies (Santa Cruz) and an ECL Western blot kit (Amersham). For Cdk4 activity, studies methodologically similar to that described for ERK were carried out at 16 h using the retinoblastoma protein as a substrate (26). For CDK1 activity, samples were obtained from cells after 36 h of incubation under various conditions, including HCC-1937 cells expressing wt BRCA1. CDK1 was immunoprecipitated using monoclonal antibody (Santa Cruz), and activity was determined by an in vitro assay with histone H1 as a substrate.

Transient transfection and constructs. Fusion plasmids encoding wt BRCA1 (pcBRCA1-385) and mutant BRCA1 proteins (the 185delAG, 5677insA, and T300G mutants; kindly provided by Michael Erdos) utilize the expression vector pcDNA3. A constitutively active MEK-1 plasmid was obtained from Upstate Biotechnology. HCC-1569 or MCF-7 cells were grown to 40 to 50% confluence and then transiently transfected with 0.5 to 10 μg of fusion plasmids, depending on the plate size and the amount of cells, with Lipofectamine reagent (GIBCO-BRL, Grand Island, N.Y.); cells were incubated with liposome-DNA complexes at 37°C for 5 h, followed by overnight recovery in 10% fetal bovine serum. Then, prior to experimental treatment, the cells were synchronized in serum-free Dulbecco's minimal essential–F-12 medium for 24 h and then treated with 17-β-E2 and/or related compounds. Cotransfections with a GFP expression vector indicated 50 to 63% efficiency of transfection. Additional plasmids included mouse ERα in pcDNA3, containing nucleotides 17 to 2001 of the steroid receptor, or the pcDNA3 backbone vector and were kindly provided by Ken Korach (5). ERK2(Y185F), a potent dominant negative construct for the mitogen-activated protein (MAP) kinase, was a kind gift from Melanie Cobb (34), and in additional studies, a small interfering RNA (siRNA) for BRCA1 was expressed. The DNA sequence against which double-stranded RNA for BRCA1 was created is 5′-TGCCAAAGUAGCTGATGTA-3′. Double-stranded RNA was transfected into MCF-7 cells, by using Oligofectamine, as 0.3 μg of siRNA/well of a six-well plate. In some studies, MCF-7 cells were transfected to express wt BRCA1, recovered, and then incubated with actinomycin D 6 h prior to the incubation with E2. ERK activity was then determined over time.

Proliferation studies. HCC-1937 cells were transfected to express ERα with or without wt BRCA1 alone or with dominant negative ERK2(Y185F). The cells were recovered overnight in serum and synchronized without serum for 12 to 24 h. The cells were then incubated in 0.2% serum (to prevent apoptosis of control cells) with or without 10 nM E2 and other substances added daily in fresh medium for 72 h, trypsinized, and counted with a Coulter counter or hemocytometer. Viability was determined by trypan blue exclusion analysis, and the counts were adjusted. Some experiments used MCF-7 or ZR-75-1 cells transfected with mutant or wt BRCA1. In additional studies, the E domain of ERα was transiently expressed and targeted in HCC-1937 cells to either the nucleus (E-Nuc-ECFP) or the plasma membrane (E-Mem-ECFP) (13) as previously described (31, 32). Proliferation was also detected in MCF-7 cells by bromodeoxyuridine (BrdU) labeling. After 24 h of treatment with 10 nM E2, the cells were incubated for 1 h with BrdU (dilution, 1:100) according to the manufacturer's protocol (Zymed, South San Francisco, Calif.). The cells were then fixed with 70% ethanol, and the incorporated BrdU was detected by an indirect immunoperoxidase method (Amersham, Arlington Heights, Ill.). Briefly, the cultured cells were incubated for 1 h with biotin-linked, mouse anti-BrdU antibody. After being washed in 20 mM Tris–500 mM NaCl–0.05% Tween 20 solution (pH 7.5), the cells were further incubated with biotinylated goat anti-mouse immunoglobulin for 10 min. The cells were then washed and incubated with peroxidase conjugates for 10 min at room temperature, and immunoreactivity was revealed by the addition of chromogen as a substrate. The cells were counterstained with hematoxylin, and the BrdU-labeled cells were counted. The study was repeated twice.

Cell cycle and immunofluorescence studies. The cell cycle distribution of the cells was determined after exposing MCF-7 cells to 10 nM E2 for 16 h (G_1/S) and 36 h (G_2/M). The cells were stained with propidium iodide, and the distribution was determined by fluorescence-activated cell sorting (FACS). For the localization of cyclin B1 during the G_2/M transition, MCF-7 cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Indirect immunofluorescent confocal microscopy was carried out with a monoclonal antibody to cyclin B1 and a fluorescein isothiocyanate-conjugated second antibody.

Western blot analysis. Immunoblot analyses of cell lysates were carried out for AKT, cyclins D1 and B1, or MKP-1 with monoclonal antibodies after the lysates were exposed to E2 for 15 min (AKT), 16 h (cyclin D1), or 36 h (cyclin B1 and MKP-1) as described previously (31, 32). Proteins were detected with an ECL Western blot kit (Amersham).

MKP-1 studies. HCC-1937 cells were transfected to express ERα plus pcDNA3 or wt BRCA1, recovered, and synchronized. The cells were then exposed (or not exposed) to 10 nM E2 for 9 min with or without 1 μM sodium vanadate (tyrosine phosphatase inhibitor) or 0.1 μM okadaic acid (serine/threonine phosphatase inhibitor). The latter were added 20 min prior to the addition of E2, and ERK activity was determined. In additional studies, double-stranded RNA for MKP-1 or GFP (control) was transfected into MCF-7 cells. Immunoblotting for MKP-1 was done daily with lysed cells over a 5-day period (temporal profile). Based upon the significant knockdown of MKP-1 at 72 h, we expressed in MCF-7 cells the siRNA for GFP (control) or MKP-1, recovered the cells over 24 h, and then expressed wt BRCA1. The cells were again recovered and synchronized over 48 h, and then ERK activity was determined in response to 10 nM E2. The DNA sequence against which double-stranded RNA for MKP-1 (QIAGEN) was created is 5′-GGACATGCTGGATGCCTTG-3′.

Phosphatase activity assay. ERK-directed phosphatase activity was determined by modifying a phosphatase activity protocol from New England Biolabs. MCF-7 cells were grown to 80% confluence and then labeled with inorganic ^{32}P (specific activity, 100 μCi/ml). The cells were lysed, and the lysate was subjected to immunoprecipitation with agarose bead-conjugated polyclonal antibody against ERK2 (Santa Cruz). This complex was extensively washed to remove unincorporated ^{32}P. After protein determination, equal amounts of labeled ERK were used as a substrate for determining phosphatase activity under various treatment conditions. Two days earlier, a second set of MCF-7 cells had been transfected with pcDNA3 (control) or wt BRCA1 and then recovered and synchronized. The cells were then incubated with or without 10 nM E2 for 9 min. The cells were washed twice with Dulbecco's minimal essential medium, scraped, and then sonicated in phosphatase activity buffer (New England Biolabs). After centrifugation, the supernatants (100 μl) from the cells subjected to each treatment were added in separate tubes to equal aliquots of ^{32}P-labeled ERK. The mixtures were then incubated with 100 μl of phosphatase activity buffer at 37°C for 30 min. After microcentrifugation, 50 μl of each supernatant was counted in a beta-counter for released ^{32}P, reflecting the ERK-directed phosphatase activity.

Apoptosis. The influence of wt BRCA expression in MCF-7 cells on cell death was determined as previously described (29). MCF-7 cells were grown on 18-mm-diameter coverslips in 12-well culture dishes in Dulbecco's minimal essential–F-12 medium without phenol red but with 0.2% charcoal-stripped serum added. The cells were transfected with pcDNA3 or wt BRCA1 expression plasmids, recovered, and then incubated in the presence or absence of 10 nM E2 for 72 h. At the end of the incubation, the cells were washed with phosphate-buffered saline and fixed with 1% freshly prepared paraformaldehyde in phosphate-buffered saline, pH 7.4, at 40°C overnight. Apoptosis was then determined by the terminal deoxynucleotidyltransferase-stimulated incorporation of nucleotides into the 3-OH end of damaged DNA in the cell, detected by fluorescent antibodies to the nucleotides (TUNEL) with a kit from Intergen, Purchase, N.Y. For each experimental condition, 400 cells were visually scored for apoptosis and viewed by fluorescence microscopy with standard fluorescein excitation and emission filters. The study was repeated. Apoptosis was also determined by FACS detection of Annexin V binding by use of a kit (Becton-Dickinson).

RESULTS

Estrogen activation of ERK is inhibited by BRCA1. We first determined that in MCF-7 and ZR-75-1 cells E2 rapidly stimulates ERK activation (Fig. 1A), which is substantially prevented by the ER antagonist, ICI182780. It was previously shown that ERK activation results from membrane ER ligation

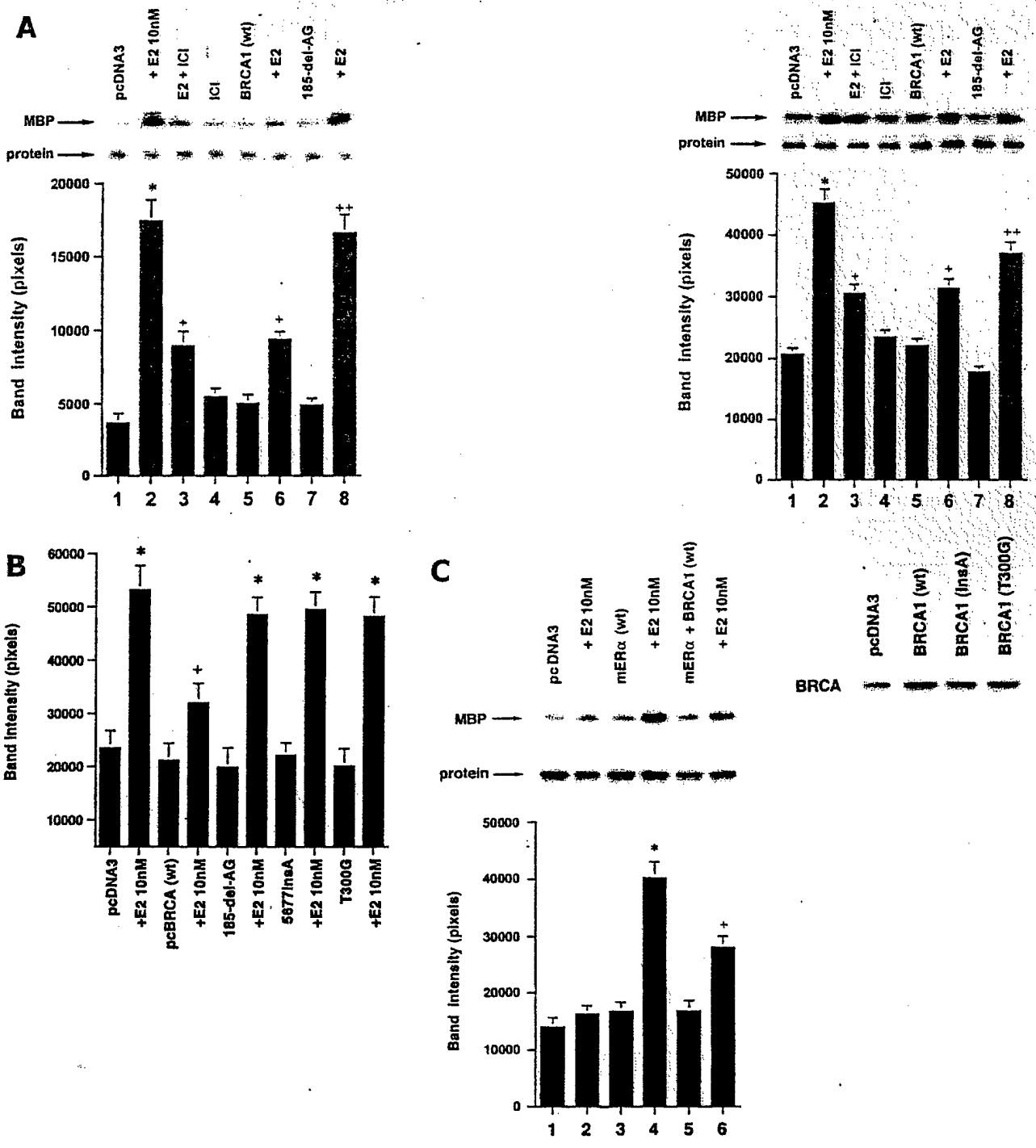


FIG. 1. wt BRCA1 but not mutant BRCA1 inhibits E2-induced ERK activation. (A) MCF-7 (left) and ZR-75-1 (right) cells, both ER positive, were transfected to express wt BRCA1 or 185delAG mutant BRCA1 or were transfected with pcDNA3 (control), recovered in serum, synchronized overnight without serum, and then incubated with 10 nM E2 for 9 min. ERK activity against myelin basic protein was then determined. ERK immunoblots are shown below activity to normalize activity for total ERK protein. The bar graph shows the combined results of three experiments. Values are means ± standard errors of the means, determined by analysis of variance plus Scheffe's test (P values of <0.05 are considered significant). Significance of results: *, P of <0.05 for pcDNA3 versus same plus E2; +, P of <0.05 for pcDNA3 plus E2 versus BRCA1 (wt) plus E2 or versus E2 plus ICI182780; ++, P of <0.05 for the 185delAG BRCA1 mutant versus same plus E2. (B) MCF-7 cells were transfected to express pcDNA3 (control), wt BRCA1, or the 185delAG, 5677insA, or T300G BRCA1 mutant, and E2-induced ERK activity was determined. Significance of results: *, P of <0.05 for pcDNA3 or mutant alone versus same plus E2; +, P of <0.05 for pcDNA3 plus E2 versus BRCA1 (wt) plus E2. (C) (Left) HCC-1937 cells were transfected with pcDNA3 or ERα, with or without a wt BRCA1 expression plasmid, and E2-induced ERK activity was determined. Significance of results: *, P of <0.05 for mouse ERα (mERa)-expressing cells versus same incubated with E2; +, P of

AQ: Y

rich4/zmb-mcb/zmb-mcb/zmb01304/zmb4182d03g | mcquinne | S=7 | 5/13/04 | 13:21 | 4/Color Fig: 1d,2f,3a,5c | Art: 1671-03

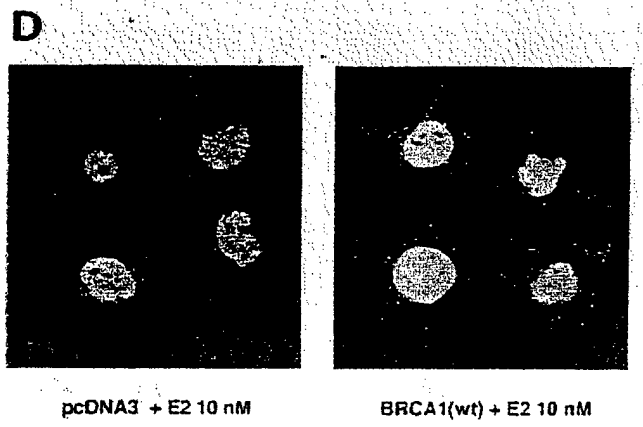
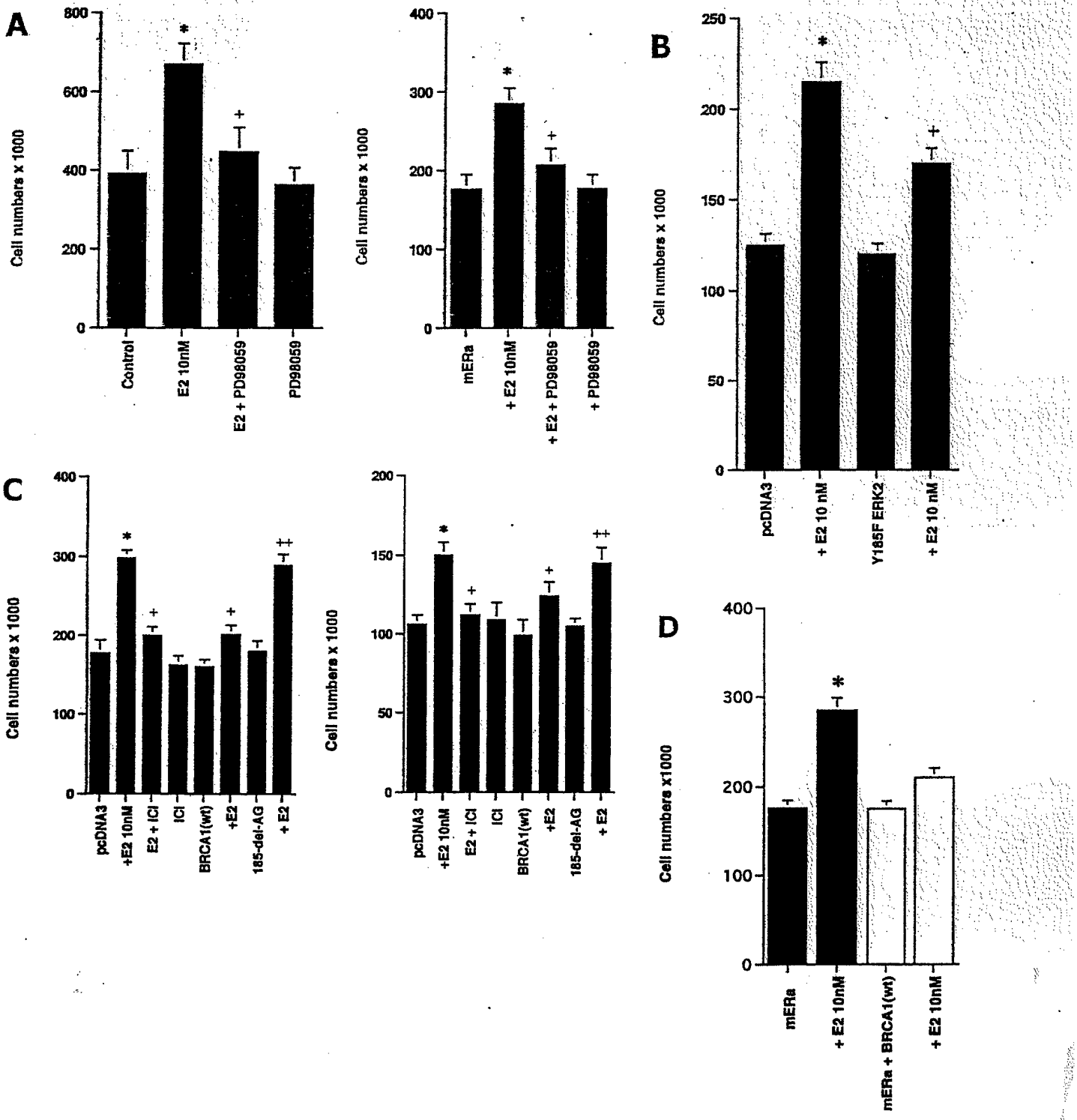


FIG. 1—*Continued*—<0.05 for ER-expressing cells plus E2 versus ER plus wt BRCA1-expressing cells plus E2. The data are from three experiments. (Right) Expression levels of wt BRCA1 and mutant BRCA1 proteins in MCF-7 cells are comparable. MCF-7 cells were transfected with pcDNA3 (control) or expression plasmids for wt BRCA1 or two mutant BRCA1 proteins, 5677insA BRCA1 (InsA) and T300G BRCA1. The cells were recovered, and 24 h later, Western blotting of the cell lysate was carried out with an N-terminal-directed antibody to BRCA1. (D) Endogenous or expressed BRCA1 localizes to the nucleus of MCF-7 cells. MCF-7 cells on coverslips were transfected with pcDNA3 or wt BRCA1, incubated with 10 nM E2 for 36 h, and then fixed for 10 min, as described in Materials and Methods. BRCA1 expression was determined by immunofluorescent confocal microscopy using a first antibody directed against the C terminus and a second antibody conjugated to fluorescein isothiocyanate. The study was repeated twice. (E) Endogenous BRCA1 restrains E2/ER signaling to ERK. (Top) Time course of BRCA1 protein knockdown by siRNA. MCF-7 cells were transfected to express an interfering RNA for BRCA1 or GFP (control), and BRCA1 protein knockdown was determined by Western blotting. (Bottom) Expression of siRNA for BRCA1 augments E2-induced ERK. MCF-7 cells transfected to express siRNA for BRCA1 or GFP were incubated 72 h later with 5 nM E2, and ERK activity was determined. wt BRCA1 was also cotransfected under one condition. A bar graph reflecting the combined results of three experiments is shown. Significance of results: *, P of <0.05 for siRNA-GFP versus same plus E2; **, P of <0.05 for siRNA-BRCA1 versus same plus E2; $^{+}$, P of <0.05 for siRNA-BRCA1 plus E2 versus same plus wt BRCA1.

by E2 (31). Expression of wt BRCA1 also inhibited ERK activation. In contrast, transfection of DNA for 185delAG BRCA1, a null mutant protein found in women with breast cancer, did not affect E2-induced ERK. Expression of two other mutant BRCA1 proteins from women with familial breast cancer, 5677insA BRCA1 and T300G BRCA1, also failed to significantly alter E2-induced ERK (Fig. 1B). This failure occurred despite the fact that wt and mutant BRCA1 proteins were comparably expressed after transfection in MCF-7 cells (Fig. 1C, right). We also examined HCC-1937 cells that have an endogenous 5382insC mutation in BRCA1 (45) and lack ER. We expressed ERα with and without wt BRCA1 in the cells to levels of protein(s) comparable to those of wt MCF-7 cells (data not shown). The ability of E2 to stimulate ERK in the ER-transfected cells was significantly inhibited when wt BRCA1 was coexpressed (Fig. 1C, left). Finally, we determined that endogenous BRCA1 produced in MCF-7 cells (Fig. 1D, left) localized to the nucleus, as did expressed wt BRCA1 (Fig. 1D, right).

We then asked whether endogenous wt BRCA1 restrains E2 signaling to ERK. First, MCF-7 cells were transfected with double-stranded RNA oligomers to BRCA1 or GFP (control), and BRCA1 protein knockdown was determined over 96 h. The siRNA for BRCA1 (and not the siRNA for GFP) produced significant inhibition of endogenous BRCA1 expression (Fig. 1E, top). We then examined the effects of this siRNA on E2-induced ERK. E2 (5 nM) stimulated ERK activity in MCF-7 cells transfected with siRNA to GFP (Fig. 1E, bottom). However, this effect was enhanced in cells transfected with the siRNA to BRCA1. When wt BRCA1 was transfected, it reversed the effects of the siRNA and restored E2-induced ERK. This suggests a novel role for endogenous, intact BRCA1, to

rich4/zmb-mcb/zmb-mcb/zmb01304/zmb4182d03g | mcquinne | S=7 | 5/13/04 | 13:21 | 4/Color Fig: 1d,2f,3a,5c | Art: 1671-03

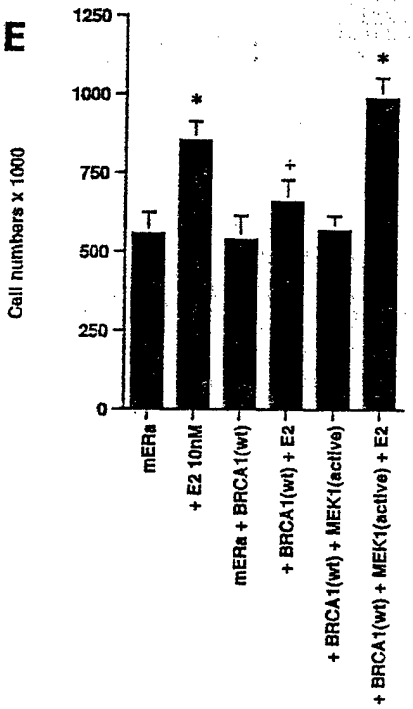


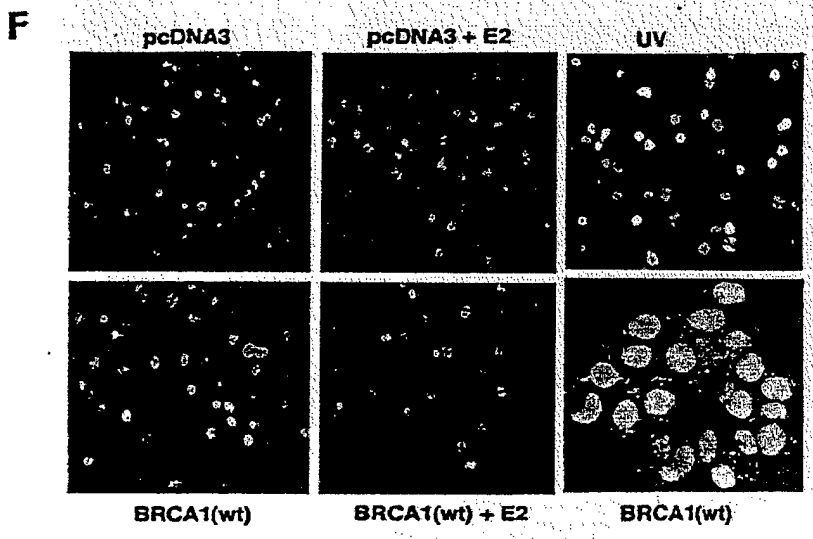
restrain ERK activation by E2. Overall, wt but not mutant BRCA1 proteins inhibit this signal from the membrane ER.

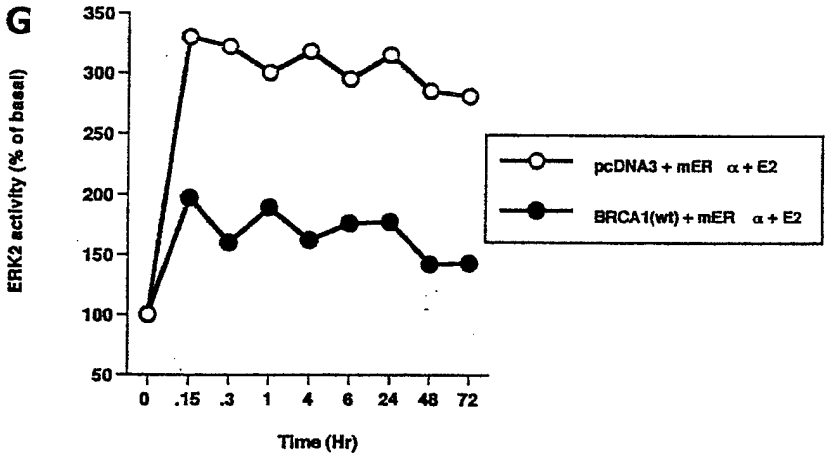
Estrogen and BRCA1 modulate ERK-induced cell proliferation. In order to understand the potential importance of the ability of wt BRCA1 to inhibit E2-induced signaling through ERK, we cultured MCF-7 cells with 10 nM E2 with and without PD98059, a specific ERK kinase (MEK) inhibitor (25). The cells were cultured for 3 days in the absence of other exogenous growth factors but in the presence of 0.2% serum to prevent apoptosis of the control cells. After 3 days, E2 caused

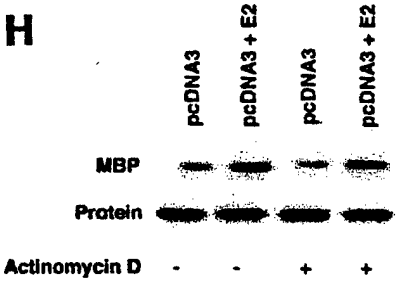
AQ: P

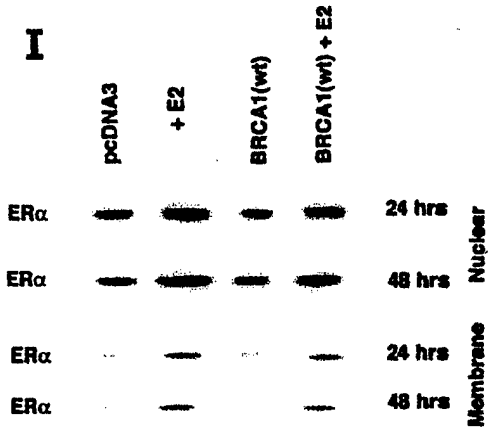
rich4/zmb-mcb/zmb-mcb/zmb01304/zmb4182d03g | mcquinne | S=7 | 5/13/04 | 13:21 | 4/Color Fig: 1d,2f,3a,5c | Art: 1671-03











a 70% increase in cell number, and this increase was 75% reversed by the MEK inhibitor (Fig. 2A, left). Similar effects occurred in ERα-expressing HCC-1937 cells (Fig. 2A, right). To corroborate the role of ERK, we expressed a dominant negative ERK2 construct (34) in the MCF-7 cells and compared steroid-induced proliferation in this setting to that in MCF-7 cells expressing the empty vector. We found that E2 was significantly less able to stimulate cell proliferation, despite a 53% efficiency of transfection of the mutant ERK2 (Fig. 2B). Thus, signaling from the membrane to ERK significantly contributes to E2-induced proliferation.

The inhibition by wt (but not mutant) BRCA1 of membrane ER signaling to ERK possibly impairs cell proliferation. To test this possibility, we carried out additional proliferation assays. E2 incubation for 3 days induced a significant increase in numbers of MCF-7, ZR-75-1, and ERα-transfected HCC-1937 cells, blocked by the ER antagonist, ICI182780 (Fig. 2C). When wt BRCA1 was transfected, MCF-7 (Fig. 2C, left) and ZR-75-1 (Fig. 2C, right) cells underwent a significant reduction in E2-related proliferation. In contrast, transfection of the 185delAG BRCA1 mutant did not significantly alter E2-induced proliferation. Similarly, E2-treated HCC-1937 cells transfected with ERα alone were greater in number than cells expressing both ERα and wt BRCA1 (Fig. 2D). To support the idea that wt BRCA1 acts through the suppression of ERK, we expressed a constitutively active MEK-1 construct that had been validated previously (30). MEK-1 directly stimulates ERK activity. Active MEK-1 reversed the ability of BRCA1 to inhibit E2-induced cell proliferation in transfected HCC-1937 cells (Fig. 2E). At this level of expression, active MEK did not by itself induce cell proliferation; it did so only in the setting of E2.

It is conceivable that wt BRCA1 expression induced cell death in the setting of E2 exposure. To test this possibility, MCF-7 cells on coverslips were transfected to express pcDNA3 or wt BRCA1, recovered, and then incubated with 10 nM E2 for 72 h. As shown in Fig. 2F, wt BRCA1 expression alone caused less than 5% cell death compared to that caused by cells incubated with 0.2% serum (pcDNA3, control) or with E2. Most importantly, we could detect an occasional cell undergoing cell death only when wt BRCA1-expressing cells were incubated with E2. As a positive control, UV exposure induced significant cell death, shown by TUNEL staining. Similar results were determined by FACS analysis of Annexin V staining (data not shown). Thus, wt BRCA1 inhibits the growth but does not induce the death of E2-treated MCF-7 cells.

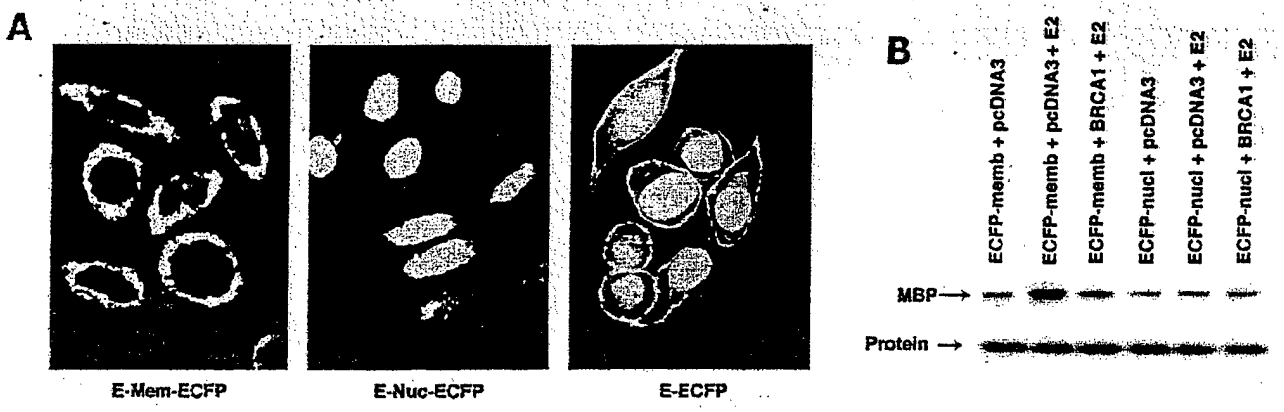
We then determined the kinetics of E2-induced ERK and its regulation by BRCA1 over 72 h. E2-induced ERK was rapidly upregulated by 9 min (first point assessed) and slightly declined over the next 50 min but remained significantly increased during the 3-day study. wt BRCA1 always suppressed E2-induced ERK activity by at least 70% (Fig. 2G). These results are consistent with a role of ERK in the interactions of E2 and BRCA1, to modulate cell proliferation over the same time period.

We also determined whether the inhibition of transcription modulated E2-induced ERK (Fig. 2H). We found that signaling by the steroid at 9 min was unaffected by 6 h of pretreatment with 4 μM actinomycin D. These results are consistent with our previous reports: targeting the E domain or full-length ERα to the cell membranes of previously ER-negative breast cancer or CHO cells results in E2-induced ERK activity. In contrast, targeting of these constructs to the nucleus does not support this signaling (30, 32). Finally, we asked whether BRCA1 might downregulate ER expression, leading to the inhibition of E2 signaling. Endogenous ERα expression was determined by Western blot analysis of both nuclear and membrane compartments of MCF-7 cells after isolation of the fractions by sucrose gradient centrifugation (25, 31). E2 caused the moderate stimulation of ERα protein expression in both compartments, but this stimulation was unaffected by BRCA1 expression at both 24 and 48 h during E2 incubation (Fig. 2I).

E domain of membrane and nuclear ERα contributes to cell proliferation. Targeting the ligand binding domain (E domain)

FIG. 2. ERK activation by E2 is important for cell proliferation. (A) MCF-7 (left) or HCC-1937 (right) cells, the latter transfected to express ERα, were incubated with 0.2% serum (control) with or without 10 nM E2 and with or without the MEK-1 inhibitor PD98059. After 3 days, the cells were trypsinized and counted. The bar graph represents the combined results from triplicate wells for each of two experiments. Significance of results: *, P of <0.05 for control versus E2; +, P of <0.05 for E2 versus E2 plus PD98059. (B) Dominant negative ERK2(Y185F) prevents E2-induced cell proliferation. Cells were transfected with dominant negative ERK2(Y185F) or pcDNA3 (control), recovered, and then incubated with 10 nM E2 for 3 days. The results are from three experiments ($n = 3$). Significance of results: *, P of <0.05 for control versus E2; +, P of <0.05 for E2 versus ERK2(Y185F) plus E2. (C) Cell proliferation induced by E2 is prevented by wt BRCA1. MCF-7 (left) or ZR-75-1 (right) cells were transfected with pcDNA3, wt BRCA1, or 185delAG mutant BRCA1, recovered, and incubated with 10 nM E2 for 3 days. To some wells, 1 μM ICI182780 (ER antagonist) was added ($n = 3$). Significance of results: *, P of <0.05 for control versus E2; +, P of <0.05 for E2 versus same plus ICI182780 or E2 plus wt BRCA1. ++, P of <0.05 for BRCA1 (wt) plus E2 versus 185delAG mutant BRCA1 plus E2. (D) wt BRCA1 expression inhibits E2-induced proliferation in HCC-1937 cells. These cells were transfected with mERa plus pcDNA3 or wt BRCA1 and incubated with 10 nM E2 for 3 days. (E) Active MEK-1 reverses BRCA1 inhibition of E2-induced proliferation. HCC-1937 cells were transfected with mERa plus pcDNA3, or mERa plus wt BRCA1, with and without active MEK-1 ($n = 3$). Significance of results: *, P of <0.05 for control versus E2, or ERα plus BRCA1 (wt) plus MEK-1 versus same in the presence of E2 (last two bars); +, P of <0.05 for E2 versus same plus BRCA1 in the presence of ERα for both. (F) wt BRCA1 does not induce apoptosis of E2-treated MCF-7 cells. MCF-7 cells were transfected to express pcDNA3 or wt BRCA1 and then recovered and incubated with and without 10 nM E2 for 72 h. Other cells were exposed to UV irradiation (50 J over 2 min). BRCA1 immunostaining revealed a 60% transfection efficiency. Apoptosis was determined by TUNEL staining. (G) Temporal kinetics of E2-induced ERK activity. HCC-1937 cells were transfected with mERa without (open circles) or with (closed circles) wt BRCA1. E2 was added daily in fresh medium, and ERK activity, normalized for ERK protein, was determined over 72 h. (H) ERK activation by E2 is unaffected by transcription inhibition. MCF-7 cells were transfected with a wt BRCA1 expression plasmid, recovered, and then incubated with the transcription inhibitor actinomycin D, at 4 μM, for 6 h prior to the addition of 10 nM E2. The cells were subsequently processed for ERK activity at 9 min after E2 addition. Total ERK2 protein is shown in this representative study of two experiments. (I) ERα protein is not modulated by wt BRCA1 expression. MCF-7 cells were transfected to express pcDNA3 (control) or wt BRCA1, recovered, and then incubated with 10 nM E2 for 24 or 48 h. Membrane and nuclear fractions were isolated by sucrose gradient centrifugation, and Western blotting was carried out using the H-222 antibody (directed against the ligand binding domain).

rich4/zmb-mcb/zmb-mcb/zmb01304/zmb4182d03g | mcquinne | S=7 | 5/13/04 | 13:21 | 4/Color Fig: 1d,2f,3a,5c | Art: 1671-03



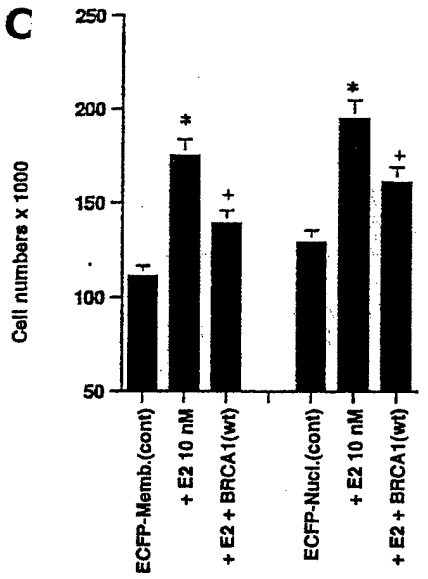
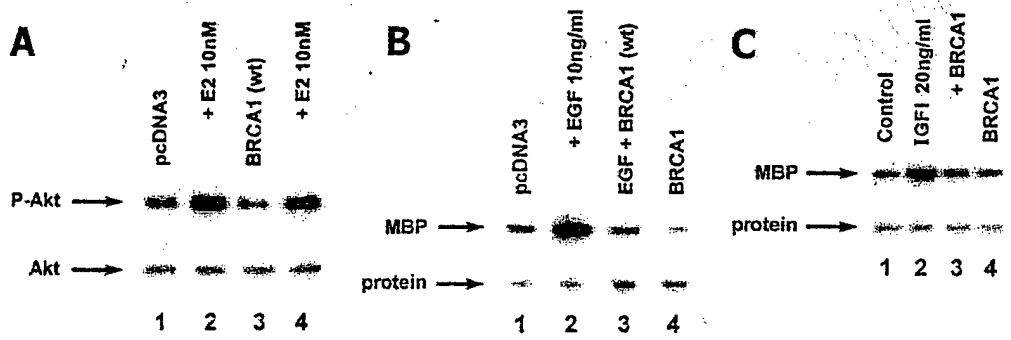


FIG. 3. (A) Distribution in HCC-1937 cells of the transfected E domain of ERα, targeted to the membrane (E-Mem-ECFP) or nucleus (E-Nuc-ECFP), or nontargeted (E-ECFP), as revealed by immunofluorescent confocal microscopy. Note that the membrane-targeted E domain appears only at the cell surface, while the nucleus-targeted E domain is localized only to the nucleus. (B) The membrane-targeted but not the nucleus-targeted E domain of ERα supports E2-induced ERK. HCC-1937 cells were transfected with one of two targeted E-domain vectors, pcDNA3 or wt BRCA1, recovered overnight, and then treated with 10 nM E2 for 9 min. ERK activity was then determined. The results of a representative study, repeated twice, are shown. (C) Membrane- and nucleus-targeted E domain contributes to E2-induced cell proliferation. Transfected HCC-1937 cells were recovered and then synchronized in the absence of serum or E2 for 16 h. The cells were then exposed to 0.2% serum plus 10 nM E2 for 3 days. Some cells were transfected to also express wt BRCA1. The bar graph represents the combined results of three experiments. Significance of results: *, P of <0.05 for control versus E2; +, P of <0.005 for E2 versus same plus BRCA1.

of ERα to the plasma membrane (but not the nucleus) of ER-negative breast cancer cells allows E2-induced ERK activation (31, 32) and the rescue of osteoblasts and HeLa cells from apoptosis (13). Rapid signaling to ERK by E2 also results in neuronal cell survival (39). We speculate here that the E domain is sufficient for E2-induced signaling from the membrane to cell proliferation. However, this does not preclude a contribution to cell proliferation by a separate action of the nuclear ER. The effects of discrete pools of ER on the stimulation of breast cancer cell proliferation have not been previously compared, and the ability of BRCA1 to prevent the two pools of ER from inducing cell proliferation is unknown.

AQ: R We therefore modeled this hypothesis by targeting the E domain of ERα to the plasma membrane or nucleus of HCC-1937 cells. The nonoverlapping cell localization of the two F3 constructs is shown in Fig. 3A. The membrane-targeted E domain reflects this portion of ERα localized to the cytoplasmic face of the membrane, with a small amount of expression perhaps taking place in ribosomes. Ligand binding studies using sucrose gradient-isolated cell compartments confirm that there is a paucity of ER in the cytoplasmic fractions and none in the nucleus. In contrast, the nucleus-targeted E domain is found exclusively in the nucleus. In our first studies, we found that only the membrane-targeted E domain of ERα supported E2-induced ERK (Fig. 3B). This effect was substantially inhibited by coexpression of wt BRCA1. We then carried out proliferation studies. E2 induced a significant increase in the number of cells expressing either the membrane- or nucleus-targeted E domain (Fig. 3C). Significantly, wt BRCA1 expression prevented E2-induced proliferation of cells expressing either membrane- or nucleus-targeted E domain. Expression of BRCA1 in the absence of E2 had little effect on the number of cells (data not shown).

BRCA1 selectively affects signaling to cell proliferation. Several molecules, including the PI3K/AKT pathway (9), often mediate cell proliferation that results from signaling initiated at the membrane. As a function of PI3K, E2 stimulates the activating phosphorylation of AKT on threonine 473 (Fig. 4A), F4 consistent with our previous findings (24). This pathway contributes to E2-induced cell cycle events (see below). However, BRCA1 expression does not affect PI3K/AKT activity, indicating a relatively specific function of BRCA1, to block ERK activity-related proliferation.



AQ: Z FIG. 4. BRCA1 does not block E2-induced AKT activity. (A) MCF-7 cells were transfected to express pcDNA3 or wt BRCA1 and recovered, and E2-induced AKT activation and phosphorylation on serine 473 were determined by immunoblotting after 15 min of incubation. Protein loading of total AKT is shown below the activity immunoblot; the study was repeated twice. (B) EGF-induced ERK is inhibited by wt BRCA1 expression. MCF-7 cells were transfected to express pcDNA3 (control) or wt BRCA1 and then treated with 10 ng of EGF/ml for 9 min, and ERK activity was determined. (C) IGF-I-induced ERK in MCF-7 cells is inhibited by BRCA1. MCF-7 cells were transfected to express pcDNA3 or wt BRCA1. After recovery, the cells were incubated with IGF-I at 20 ng/ml for 9 min, and ERK was determined. The results from a representative study, repeated twice, are shown.

BRCA1 inhibits growth factor signaling to proliferation. Signaling to ERK by other important breast cancer growth factors, such as EGF and IGF-I, rapidly occurs after these proteins bind specific tyrosine kinase growth factor receptors in the plasma membrane. Activation of ERK by these growth factor receptors is also important for cell proliferation (12, 43). We therefore asked whether BRCA1 could also inhibit this mechanism of EGF or IGF-I to stimulate breast cancer proliferation.

We first determined that BRCA1 prevents EGF-induced ERK activation (Fig. 4B). This action coincided with the ability of BRCA1 to suppress EGF-induced proliferation of HCC-1937 cells (Table 1). The native cells express EGFR but not ER and respond to EGF (but not E2) with significantly increased proliferation (Table 1). EGF action was substantially reversed by tyrphostin AG1478, a specific inhibitor of EGFR tyrosine kinase activity. In cells transfected to express ERα, E2 induced a strong proliferative response that was modestly enhanced by coincubation with EGF (Table 1). The lack of enhanced proliferation in response to the combination of E2 and EGF may reflect the fact that E2 strongly induces EGFR transactivation (8, 32) so that the addition of EGF is not significant. Notably, the proliferative responses here to only EGF or E2 were nearly identical. We also found that proliferation in response to E2 was inhibited by tyrphostin AG1478 (Table 1). In contrast, addition of tyrphostin to the platelet-derived growth factor receptor kinase activity had no influence on E2-induced proliferation (data not shown). Thus, membrane ER cross talk to the EGFR is essential for ERK activation (31) and breast cancer cell proliferation, as shown in the present study. We also found that wt BRCA1 expression inhibits E2-, EGF-, or E2-plus-EGF-induced proliferation, consistent with the ability of the tumor suppressor to downregulate ERK signaling. Finally, we investigated the interactions of BRCA1 and IGF-I. IGF-I stimulated ERK activity (Fig. 4C) and cell proliferation (data not shown), and these actions were prevented by wt BRCA1 expression. Thus, wt BRCA1 broadly suppresses signaling by established mitogens in breast cancer cells, a novel action.

T1 AQ: S

TABLE 1. Interactions of estradiol and EGF with BRCA1 in regulating HCC-1937 cell proliferation[a]

Expression vector	Condition	No. of cells (10^3)
pcDNA3	Control	148 ± 15
	E2 (10 nM)	158 ± 17
	EGF (10 ng/ml)	227 ± 20[b]
	EGF + tyrphostin AG1478	154 ± 17[c]
	EGF + wtBRCA1	171 ± 19[c]
	Tyrphostin AG1478	152 ± 17
ERα	Control	134 ± 14
	E2 (10 nM)	211 ± 21[b]
	E2 + EGF	227 ± 18[b]
	E2 + tyrphostin AG1478	160 ± 16[c]
	EGF	212 ± 19[b]
	EGF + tyrphostin AG1478	127 ± 15[c]
	E2 + EGF + wtBRCA1	174 ± 17[c]
	EGF + wtBRCA1	170 ± 15[c]
	wtBRCA1	124 ± 15

[a] Cells were transfected to express pcDNA3 or ERα with and without wtBRCA1 and incubated with E2 or EGF and tyrphostin AG1478 (EGFR tyrosine kinase inhibitor) for 3 days. Data are means ± standard errors of the results of three experiments combined, with triplicate determinations for each condition in each experiment.

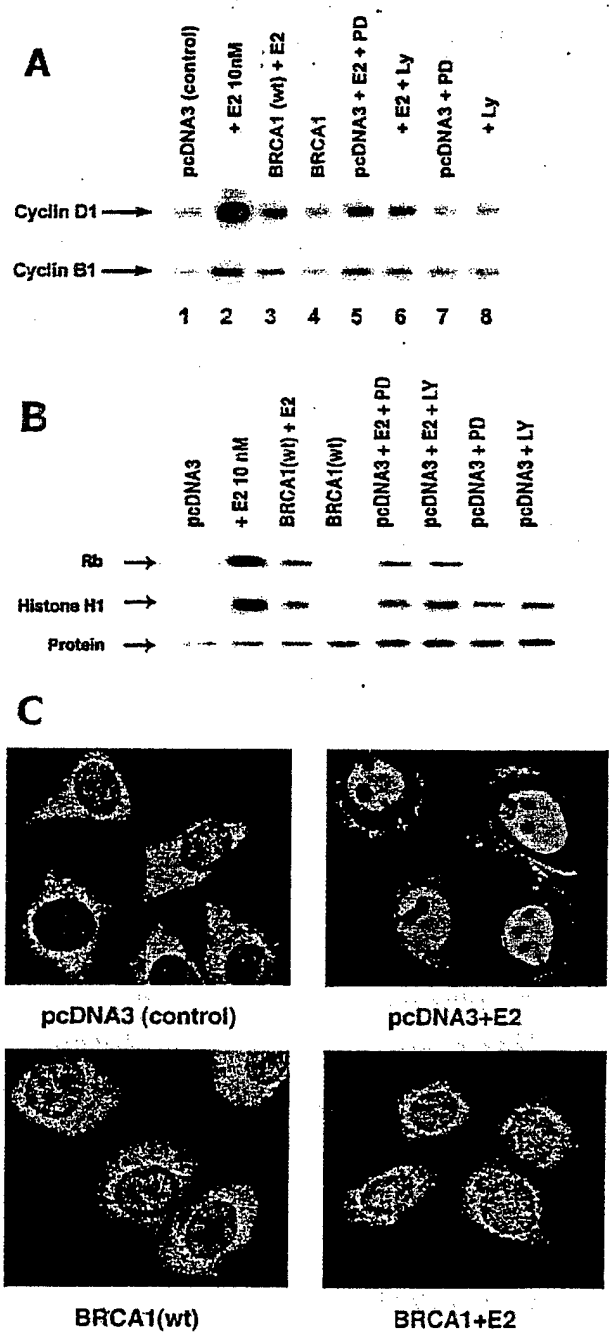
[b] P of <0.05 for control versus treated cells as determined by analysis of variance plus Scheffe's test.

[c] P of <0.05 for cells treated with E2 or EGF versus the same plus tyrphostin or BRCA1.

E2 induces G_1/S and G_2/M cell cycle progression that is prevented by BRCA1. What proliferation-related actions of E2 are dependent on membrane ER signaling through ERK and are opposed by wt BRCA1? The ability of E2 to induce cyclin D1 production is significantly dependent upon ERK activation in breast cancer (30). Cyclin D1 upregulates Cdk4 activity that contributes to the inactivating phosphorylation of the retinoblastoma protein, leading to G_1/S-phase cell cycle progression. This is considered to be important for the proliferation of breast cancer (51). Here, we found that E2 induces cyclin D1 protein in MCF-7 cells and that expression of wt BRCA1 significantly prevents this (Fig. 5A). E2 stimulates cyclin D1 through ERK and PI3K-related actions since the effects of E2 were partially reversed by PD98059 (MEK inhibitor) and LY294002 (PI3K inhibitor). Cdk4 activity was also induced by E2 via ERK and PI3K actions, and this was substantially reduced by BRCA1 expression (Fig. 5B). These results were corroborated by FACS analysis showing that the number of

F5

rich4/zmb-mcb/zmb-mcb/zmb01304/zmb4182d03g | mcquinne | S=7 | 5/13/04 | 13:21 | 4/Color Fig: 1d,2f,3a,5c | Art: 1671-03



F6

FIG. 5. BRCA1 inhibits key cell cycle actions induced by E2. (A) MCF-7 cells were transfected with wt BRCA1 or pcDNA3 (control) and then incubated with or without 10 nM E2 for 16 h (cyclin D1) or 36 h (cyclin B1). For some cells, 1 μM PD98059, a MEK inhibitor, or 10 μM LY294002, a PI3K inhibitor, was added. Cyclins were detected by Western blotting. (B) MCF-7 cells were incubated with E2 after recovery from transfection. Cdk4 activity was determined after 16 h of incubation by immunoprecipitating the kinase from cell lysates and adding a portion to an in vitro activity assay utilizing retinoblastoma protein as the substrate. CDK1 activity was determined after 36 h of incubation by an assay with histone H1 as the substrate. CDK1 total protein blots are shown below each sample. The results of three experiments are represented. (C) Transfected MCF-7 cells were incubated with or without E2 for 36 h. Cyclin B1 cell distribution was determined by immunofluorescent microscopy during G_2/M.

cells in S phase doubled after 16 h of E2 incubation. The passage of cells into S phase was reduced by 60% by BRCA1 transfection or 70% by incubation with PD98059.

Regarding the G_2/M checkpoint, the cyclin B1 protein controls the activity of CDK1 kinase, which is necessary for passage into M phase (27). We found that E2 induces increased cyclin B1 protein levels via both ERK and PI3K and that wt BRCA1 expression partially prevents this (Fig. 5A). E2 also induced CDK1 activity (Fig. 5B), which is significantly prevented by wt BRCA1 expression. BRCA1 has previously been reported to downregulate cyclin B1 production through unknown mechanisms that impact gene regulation. To further understand the interactions between E2/ER and BRCA1 during the critical G_2/M transition, we determined the subcellular localization of cyclin B1. Cyclin B1 is diffusely distributed throughout the cytoplasm in pcDNA3-transfected MCF-7 cells (Fig. 5C). Expression of wt BRCA1 in the absence of E2 similarly results in typical cytoplasmic localization of cyclin B1. Following 36 h of incubation with E2, cyclin B1 translocates substantially to the nucleus (Fig. 5C, upper right). However, when wt BRCA1 is expressed and these cells are incubated with E2, cyclin B1 remains predominantly cytoplasmic. Thus, wt BRCA1 inhibits E2-induced cell cycle events that are essential to G_1/S and G_2/M progression.

BRCA1 induces a specific ERK phosphatase. We have established that BRCA1 inhibits E2 signaling through ERK and that this is important for the growth regulatory actions of the tumor suppressor. wt BRCA1 is predominantly a nuclear protein and sometimes transactivates genes or stabilizes proteins that contribute to its role as a tumor suppressor (37). Therefore, we postulated that BRCA1 induces a phosphatase with strong activity against nuclear ERK. To test this idea, we determined whether the ability of expressed wt BRCA1 to inhibit ERK in ER-transfected HCC-1937 cells was dependent on phosphatase activity. BRCA1 inhibited E2-induced ERK, but this inhibition was partially reversed by either tyrosine or threonine/serine phosphatase inhibitors (Fig. 6A). Phosphatase inhibitors or BRCA1 alone had little effect when ERK activity was normalized for protein. We also found similar results for the reversal of BRCA1 inhibition of EGF- or IGF-I-induced ERK activity (data not shown).

We hypothesized that BRCA1 might induce a dual-specificity phosphatase with activity against ERK, such as MKP-1 (4). We speculated that at 9 min, BRCA1 upregulates ERK-directed phosphatase activity and modulates the longer-term inhibition of kinase through the induction of MKP-1 protein. We first determined phosphatase activity directed against ^{32}P-labeled ERK. E2 (10 nM) alone significantly reduced ERK-directed phosphatase activity in the MCF-7 cell lysate after 9 min of incubation, probably contributing to the activation of this kinase by the steroid (Fig. 6B). In contrast, BRCA1 substantially reversed this action of E2, correlating with the acute inhibition of E2-induced ERK.

To assess the longer-term effect of BRCA1, MCF-7 cells transfected to express BRCA1 or pcDNA3 (control) were incubated or not incubated with E2 for 24 h. MKP-1 protein levels were then determined. BRCA1 significantly induced MKP-1 protein only in the setting of cotreatment with E2 (Fig. 6C). Thus, it is in the state of ERK activation (stimulated by E2) that BRCA1 upregulates MKP-1 expression. This could

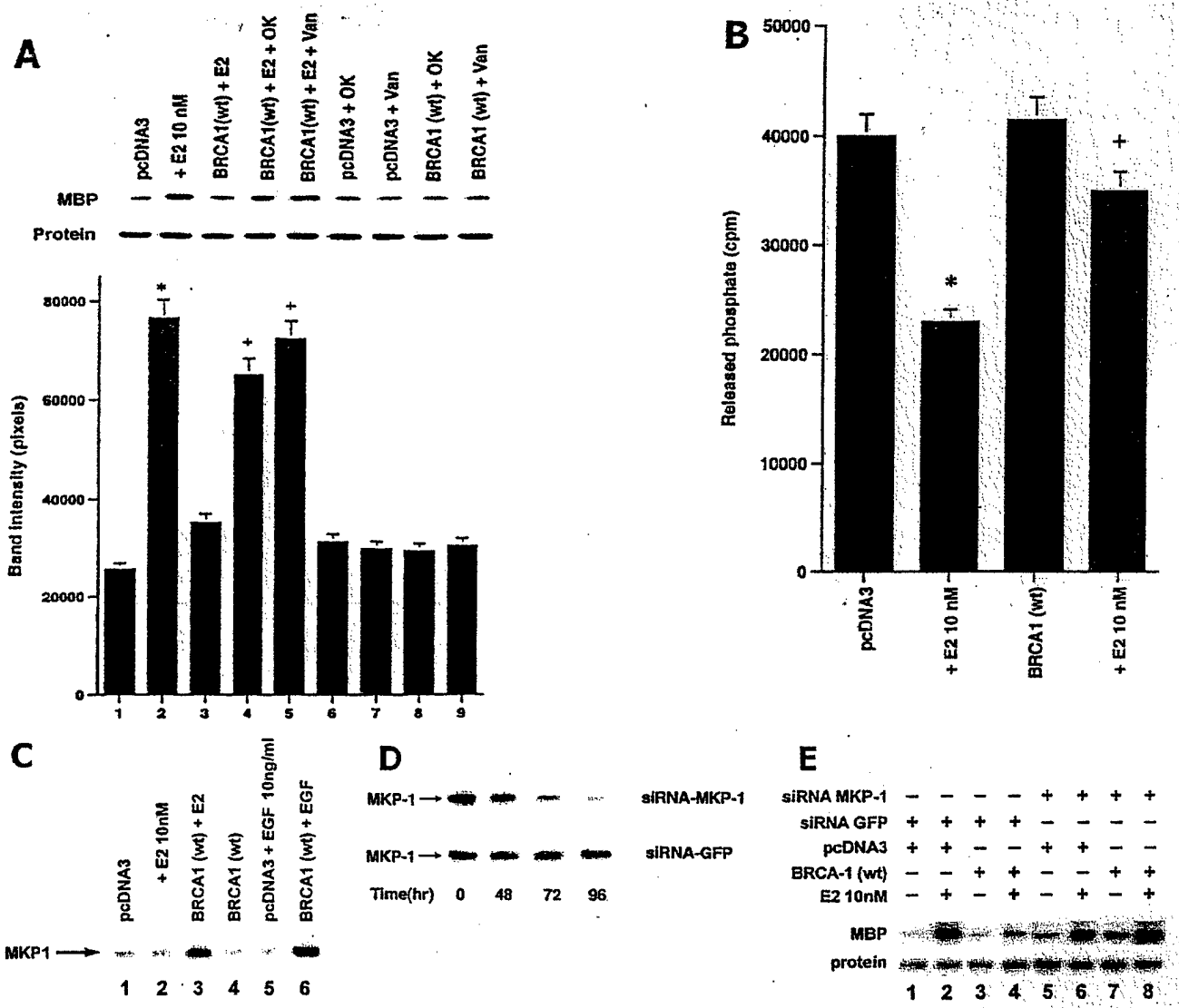


FIG. 6. BRCA1 activates phosphatase activity to downregulate ERK activity. (A) Phosphatase inhibitors reverse BRCA1 inhibition of E2-stimulated ERK. HCC-1937 cells were transfected to express ER plus pcDNA3 or wt BRCA1, recovered and synchronized for 48 h, and then exposed (or not exposed [pcDNA3, lane 1]) to 10 nM E2 for 9 min. Under some conditions, the cells were also incubated with 1 μM of sodium vanadate (Van) or 0.1 μM okadaic acid (OK) 20 min prior to E2 addition. Phosphatase inhibitors or BRCA1 alone had little effect when ERK activity was normalized for protein. The bar graph reflects the combined results of three experiments. Significance of results: *, P of <0.05 for pcDNA3 versus same plus E2; +, P of <0.05 for BRCA1 (wt) plus E2 versus same plus okadaic acid or same plus Van. (B) BRCA1 prevents E2-induced downregulation of ERK phosphatase activity. ^{32}P-labeled ERK2 was prepared as described in Materials and Methods and used as a substrate for an in vitro phosphatase assay. MCF-7 cells transfected with pcDNA3 (control) or wt BRCA1 were incubated with or without 10 nM E2 for 9 min, and the cells were then lysed. Equal protein aliquots of cell lysate were then added to ^{32}P-labeled ERK2 protein aliquots, and phosphatase activity was determined. The data are from triplicate determinations for each condition and are representative of two separate studies. (C) BRCA1 upregulates MKP-1 protein. MCF-7 cells were transfected with pcDNA3 (control) or wt BRCA1, recovered, synchronized in the absence of steroid or serum, and then incubated in medium with or without 10 nM E2 for 24 h. Western blot analyses from cell lysates were carried out for immunoprecipitated MKP-1. The representative study was repeated twice. (D) siRNA for MKP-1 downregulates MKP-1 protein. MCF-7 cells were transfected with annealed, double-stranded RNA for MKP-1 or GFP by using Oligofectamine, as described in Materials and Methods. Western blot analyses for MKP-1 were accomplished in cells lysed at 48, 72, and 96 h posttransfection. (E) siRNA for MKP-1 reverses BRCA1 inhibition of E2-induced ERK. MCF-7 cells were sequentially transfected with 0.3 μg of double-stranded RNA oligomers (to GFP or MKP-1) on day 1, recovered, and then transfected 24 h later with pcDNA3 or wt BRCA1. After recovery and synchronization over 48 h in 0.2% serum, E2 was added for 9 min. ERK activity was determined 72 h after siRNA transfection. The experiment was repeated twice. (F) siRNA for MKP-1 prevents wt BRCA1 inhibition of E2-induced proliferation. MCF-7 cells were transfected with pcDNA3 or wt BRCA1, recovered, and then transfected with siRNA for GFP or MKP-1. The cells were recovered and then incubated with 10 nM E2 for 23 h, followed by BrdU pulsing for 1 h. The cells were fixed, and BrdU labeling was detected as described in Materials and Methods. The data are the means ± standard errors of the means for results with 1,000 cells counted per condition in each of three separate experiments and then combined.

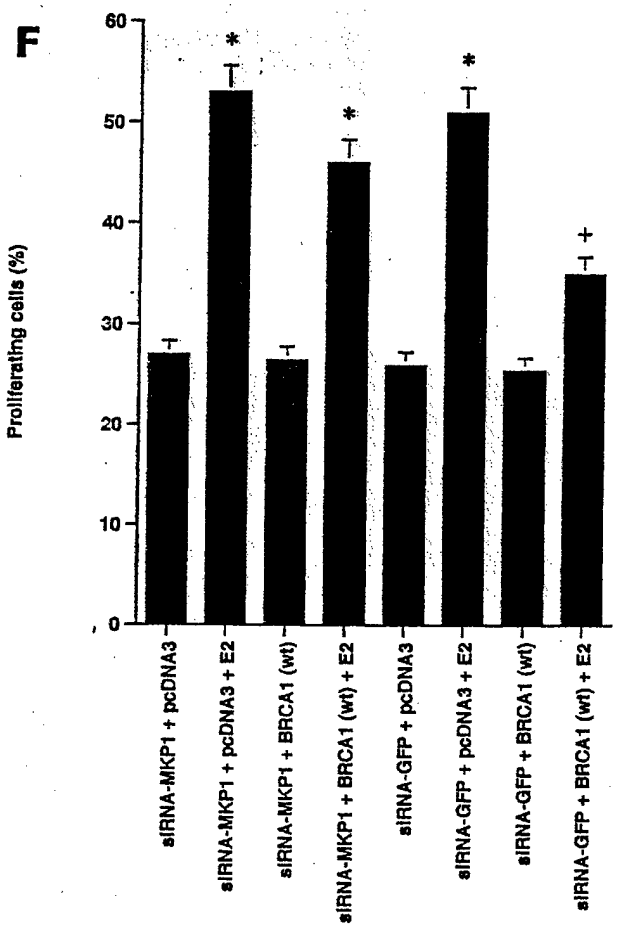


FIG. 6—*Continued.*

reflect a transcriptional upregulation of MKP-1 in the setting of both E2 and BRCA1. Comparable to the interaction with E2, BRCA1 strongly induced MKP-1 when the cells were exposed to EGF.

To specifically implicate MKP-1, we utilized an siRNA approach. We first transfected MCF-7 cells with double-stranded RNA for GFP (control) or MKP-1 to determine protein knockdown over 5 days. At 72 h, specifically the siRNA for MKP-1 lowered the expression of this protein (Fig. 6D). We then carried out ERK activation studies. Expression of the siRNA for GFP, sequentially followed by transfection of pcDNA3 (control), did not affect the strong activation of ERK by E2 (Fig. 6E). When wt BRCA1 was expressed with the siRNA for GFP, the activation of ERK by E2 was barely evident. Upon expression of the siRNA for MKP-1, followed by transfection of pcDNA3, E2 strongly activated ERK. In contrast, when the siRNA for MKP-1 and the plasmid containing wt BRCA1 were sequentially expressed, E2 activation of ERK was no longer inhibited.

We then determined whether MKP-1 knockdown also affected cell proliferation. BrdU incorporation into the MCF-7 cells was stimulated by E2 and inhibited by wt BRCA1 expression. However, in the presence of the siRNA for MKP-1 (but not the siRNA for GFP), BRCA1 inhibition of proliferation was substantially prevented (Fig. 6F). Therefore, the ability of BRCA1 to upregulate ERK phosphatase activity via the MKP-1 protein is functionally important in blocking E2-induced ERK and cell proliferation.

DISCUSSION

It is estimated that up to 80% of women expressing mutant BRCA1 ultimately develop breast cancer (21), but it is unknown why such mutations only predispose to cancer of hormonally responsive tissues. This predisposition perhaps results from a tumor-promoting interaction of mutant BRCA1 with estrogen. Here, we report the novel finding that intact BRCA1 inhibits E2 signaling to ERK and breast cancer cell proliferation. In contrast, three mutations of BRCA1 that are commonly found in women with breast cancer fail to suppress E2 signaling to proliferation. Both effects of intact BRCA1 result substantially from the ability of this tumor suppressor to induce MAP kinase phosphatase production and activity. As an additional antiproliferative mechanism, the ability of BRCA1 to block nuclear ER-induced transcription (6, 7) might also contribute. We definitively implicated MKP-1 by use of an interfering RNA approach. This finding is consistent with BRCA1 being localized mostly to the nucleus (3), where it inhibits the final step (effector) in membrane ER signaling to ERK activation and cell cycle progression. Furthermore, BRCA1-induced MKP-1 protein can accomplish the longer-term inhibition of E2-induced ERK, demonstrated in the present study over 3 days. This is relevant to breast cancer since it is the sustained ERK signaling induced by growth factors in this disease that stabilizes oncogenic proteins, such as c-fos (23).

The rapid activation of ERK stems from steroid ligation of the plasma membrane pool of ER. Membrane targeting of either (i) ERα that are deficient in the nuclear localization sequence (53) or (ii) the E domain of ERα in ER null cells (31) is sufficient for the rapid activation of this kinase by E2. In contrast, targeting of only the E domain (31, 32) or full-length receptor to the nucleus (unpublished observations) does not result in E2-induced ERK. We report here that the activation of ERK by E2 is not prevented by actinomycin D. This result suggests that an important effect of the steroid is to modulate acutely the activity of the kinase through inhibition of phosphatase activity as shown in the present study. It is also possible that E2 prolongs the survival of ERK protein, contributing to the long-term signaling by the sex steroid. We demonstrate the importance of E2 signaling to ERK activation in that E2-induced breast cancer cell proliferation is substantially prevented by (i) a soluble inhibitor of ERK kinase or (ii) expression of a dominant negative ERK2 protein. E2 signaling through ERK to cyclin D1 production and Cdk4 activity underlies the passage of breast cancer cells through G_1 to S phase of the cell cycle (30; present study). We also report the novel finding that E2-induced signaling through ERK (and PI3K) leads to cyclin B1 and CDK1 activity upregulation and passage into M phase. These cell cycle effects of E2 are prevented by BRCA1 and affirm in this setting the ability of this tumor suppressor to induce G_1/S and G_2/M checkpoint activities (40, 49). Supporting a causal relationship between BRCA1 and ERK, constitutively active MEK protein reverses BRCA1 inhibition of E2-induced proliferation.

Breast cancer cells such as MCF-7 cells typically express

both membrane and nuclear ER. In vivo, the administration of antibodies to ERα blocks the growth of human breast cancer xenografts in nude mice, presumably through the prevention of membrane ER signaling to ERK and PI3K previously demonstrated in vitro (20). In addition, ERK and PI3K signaling stimulates gene transcription that is relevant to breast cancer (18, 47). The traditional function of the nuclear ER of transactivating relevant genes in this disease is important. However, in breast cancer, an overexpressed and truncated MTA1 protein sequesters ER away from the nucleus and strongly reduces E2-activated transcription yet promotes increased ERK signaling and aggressive tumor behavior (14). These findings suggest the additional importance of nontranscriptional actions of ERK to the promotion of malignancy, and it was previously reported that E2 signaling from the membrane increases cell survival through posttranslational effects (29).

We show here the first comparison of the contributions of distinct ER pools to breast cancer proliferation. Upon targeting the E domain of ERα to the plasma membranes of HCC-1937 cells, E2 significantly stimulates cell division. This occurs despite the fact that the nuclear ER is absent. Thus, some events critical for E2-induced breast cancer cell proliferation may require only the membrane E-domain function. It was recently shown that targeting the E domain to the cell membrane of ER-negative breast cancer cells caused a series of G-protein-coupled signaling events that led to the transactivation of the EGF receptor and subsequent activation of ERK (32). Targeting the E domain to the nucleus did not support this signaling. However, targeting the E domain to the nucleus, as reported here, results in E2-stimulated growth of the tumor cells. This result supports the idea that both ER populations contribute to breast cancer cell biology and impact common key targets, such as cyclin D1 production. It is well recognized that membrane growth factor signaling can augment nuclear ER function (reviewed in reference 15), which occurs through several mechanisms, including the activating phosphorylation of nuclear ER and the recruitment of coactivator proteins. Since membrane ER transactivates EGFR and ErbB2 (8, 32, 41), we propose an integrated model wherein membrane ER signaling though the tyrosine kinase growth factor receptors augments nuclear ER function in MCF-7 cells, thereby promoting proliferation. This proliferation occurs in addition to the direct effects of kinase activation that modify the functions of existing proteins and transactivate genes (24). As determined here, BRCA1 inhibits proliferation arising from either E-domain model.

An important finding is that intact BRCA1 also prevents EGF and IGF-I signaling through ERK to cell proliferation. EGF and EGF-1 are strongly implicated in the biology of human breast cancer, where they signal through this member of the MAP kinase family to cell growth (27, 50). EGF serves as a ligand for important heterodimers of the EGFR family, including the EGFR/ErbB2 heterodimer that is implicated in breast cancer pathogenesis or aggressiveness. It was recently demonstrated that E2 activates ERK in breast cancer via transactivation of the EGF receptor (8, 32). We find here that a specific EGFR tyrosine kinase inhibitor strongly prevents E2-induced cell proliferation. Thus, the ability of BRCA1 to inhibit both membrane ER and EGFR-induced ERK activation and subsequent proliferation is consistent with a functional role of this cross talk between steroid and growth factor receptors. There is also abundant evidence that ER/IGF-I receptor cross talk participates in the biology of this malignancy (reviewed in reference 15). Thus, in ER-positive cells, the ability of intact BRCA1 to oppose individual and collective signaling from the membrane is likely to be important as a tumor suppressor mechanism.

Yan et al. recently reported that BRCA1 overexpression in MCF-7 cells results in JNK and ERK activation (50). The latter signaling contributed to cell survival of BRCA1, only in MCF-7 cells. However, those investigators did not determine the interactions between E2 and BRCA1 nor the effects of mutant BRCA1. Different subsets of MCF-7 cells have been identified, and a minority actually respond to estrogen with cell death. We find that BRCA1 expression stimulates JNK and significantly induces apoptosis in MCF-7 cells only during an additional stress (UV radiation or paclitaxel); this stimulation is inhibited by E2 (unpublished observations). Here we show that BRCA1 does not stimulate apoptosis, particularly when E2 is present. BRCA1 inhibits ERK as induced by E2 and growth factors for as long as 72 h. This finding is consistent with a tumor-suppressive action of BRCA1. We further report that BRCA1 inhibits E2-induced ERK in three different breast cancer cell lines, including HCC-1937 cells transfected to express intact BRCA1, which represents a nonoverexpression model for BRCA1 action.

Estrogen use is a moderate risk factor for the development of breast cancer in women (35). This is attributed to the ability of the sex steroid to promote cell proliferation and survival and underlies the rationale for using tamoxifen, a drug that prevents the in vivo development or recurrence of ER-positive breast cancer (43). BRCA1 may serve as an endogenous restraint on both steroid and growth factor signaling to proliferation in women, the majority of whom have intact BRCA1. We propose that the loss of signaling restraint due to BRCA1 mutation might be a determining stimulus that promotes the development of breast cancer. This possibility could be relevant to both ER-positive and ER-negative tumor development, as the latter is dependent upon growth factor receptor signaling.

ACKNOWLEDGMENTS

This work was supported by grants from the Research Service of the Department of Veterans Affairs, Avon Products Breast Cancer Research Foundation, Department of Defense Breast Cancer Research Program (grant no. BC990915), and the NIH (HL-59890) (to E.R.L.), and by NCI grants CA82599 and CA800000, a Department of Defense Breast Cancer Research Program grant (BC999254), and Susan G. Komen Breast Cancer Foundation grant 99-003255S (to E.M.R.).

REFERENCES

1. **Baer, R., and T. Ludwig.** 2002. The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity. Curr. Opin. Genet. Dev. 12:86–91.
2. **Casey, G.** 1995. The BRCA1 and BRCA2 breast cancer genes. Curr. Opin. Oncol. 9:88–93.
3. **Chen, Y., A. A. Farmer, C.-F. Chen, D. C. Jones, P.-L. Chen, and W.-H. Lee.** 1996. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. Cancer Res. 56:3168–3172.
4. **Chu, Y., P. A. Solski, R. Khosravi-Far, C. J. Der, and K. Kelly.** 1996. The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. J. Biol. Chem. 271:6497–6501.
5. **Couse, J. F., S. W. Curtis, T. F. Washburn, J. Lindzey, T. S. Golding, D. B. Lubahn, O. Smithies, and K. S. Korach.** 1995. Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Mol. Endocrinol. 9:1441–1454.

6. **Fan, S., J.-A. Wang, R. Yuan, Y. Ma, Q. Meng, M. R. Erdos, R. G. Pestell, F. Yuan, K. J. Auborn, I. D. Goldberg, and E. M. Rosen.** 1999. BRCA1 inhibition of estrogen receptor signaling in transfected cells. Science **284:** 1354–1356.
7. **Fan, S., Y. X. Ma, C. Wang, R. Q. Yuan, Q. Meng, J. A. Wang, M. Erdos, I. D. Goldberg, P. Webb, P. J. Kushner, R. G. Pestell, and E. M. Rosen.** 2001. Role of direct interaction in BRCA1 inhibition of estrogen receptor activity. Oncogene **20:**77–87.
8. **Filardo, E. J., J. A. Quinn, K. I. Bland, and A. R. Frackelton.** 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, gpr30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. **14:** 1649–1660.
9. **Fruman, D. A., R. E. Meyers, and L. C. Cantley.** 1998. Phosphoinositide kinases. Annu. Rev. Biochem. **67:**481–507.
10. **Gowen, L. C., A. V. Avrutskaya, A. M. Latour, B. H. Koller, and S. A. Leadon.** 1998. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science **281:**1009–1012.
11. **Harkin, D. P., J. M. Bean, D. Miklos, Y.-H. Song, V. B. Truong, C. Englert, F. C. Christians, L. W. Ellisen, S. Maheswaran, J. D. Oliner, and D. A. Haber.** 1999. Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell **97:**575–586.
12. **Kaufmann, K., and G. Thiel.** 2002. Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. J. Cell. Biochem. **85:**381–391.
13. **Kousteni, S., T. Bellido, L. I. Plotkin, C. A. O'Brien, D. L. Bodenner, L. Han, K. Han, G. B. DiGregorio, J. A. Katzenellenbogen, B. S. Katzenellenbogen, P. K. Roberson, R. S. Weinstein, R. L. Jilka, and S. C. Manolagas.** 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell **104:**719–730.
14. **Kumar, R., E.-A. Wang, A. Mazumdar, A. H. Talukder, M. Mandal, Z. Yang, R. Bagheri-Yarmand, A. Sahin, G. Hortobagyi, L. Adam, C. J. Barnes, and R. K. Vadlamudi.** 2002. A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature **418:**654–657.
15. **Levin, E. R.** 2003. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol. Endocrinol. **17:**309–317.
16. **Loman, N., O. Johannsson, P. O. Bendahl, A. Borg, M. Ferno, and H. Olsson.** 1998. Steroid receptors in hereditary breast carcinomas associated with BRCA1 or BRCA2 mutations or unknown susceptibility genes. Cancer **83:**310–319.
17. **MacLachlan, T. K., R. Takimoto, and W. S. El-Deiry.** 2002. BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets. Mol. Cell. Biol. **22:**4280–4292.
18. **Marino, M., F. Acconcia, F. Bresciani, A. Weisz, and A. Trentalance.** 2002. Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells. Mol. Biol. Cell. **13:**3720–3729.
19. **Marks, J. R., G. Huper, J. P. Vaughn, P. L. Davis, J. Norris, D. P. McDonnell, R. W. Wiseman, P. A. Futreal, and J. D. Iglehart.** 1997. BRCA1 expression is not directly responsive to estrogen. Oncogene **14:**115–121.
20. **Marquez, D. C., and R. J. Pietras.** 2001. Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene **20:**5420–5430.
21. **Miki, Y., J. Swensen, D. Shattuck-Eidens, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L. M. Bennett, W. Ding, et al.** 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science **266:**66–72.
22. **Moynahan, M. E., J. W. Chiu, B. H. Koller, and M. Jasin.** 1999. Brca1 controls homology-directed DNA repair. Mol. Cell **4:**511–518.
23. **Murphy, L. O., S. Smith, R. H. Chen, D. C. Fingar, and J. Blenis.** 2002. Molecular interpretation of ERK signal duration by immediate early gene products. Nat. Cell. Biol. **4:**556–564.
24. **Pedram, A., M. Razandi, M. Aitkenhead, C. C. W. Hughes, and E. R. Levin.** 2002. Integration of the non-genomic and genomic actions of estrogen: membrane initiated signaling by steroid (MISS) to transcription and cell biology. J. Biol. Chem. **277:**50768–50775.
25. **Pedram, A., M. Razandi, and E. R. Levin.** 2002. Deciphering VEGF signaling to vascular permeability: inhibition by atrial natriuretic peptide. J. Biol. Chem. **277:**44385–44398.
26. **Pedram, A., M. Razandi, R.-M. Hu, and E. R. Levin.** 1998. Astrocyte progression through G1-S phase of the cell cycle depends upon multiple protein interactions. J. Biol. Chem. **273:**13966–13972.
27. **Pines, J., and T. Hunter.** 1992. Cyclins A and B1 in the human cell cycle. Ciba Found. Symp. **170:**187–196.
28. **Prenzel, N., E. Zwick, M. Leserer, and A. Ullrich.** 2000. Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. Breast Cancer Res. **2:**184–190.
29. **Razandi, M., A. Pedram, and E. R. Levin.** 2000. Estrogen signals to preservation of endothelial cell form and function. J. Biol. Chem. **275:**38540–38546.
30. **Razandi, M., G. Alton, A. Pedram, S. Ghonshani, P. Webb, and E. R. Levin.** 2003. Identification of a structural determinant necessary for the localization and function of estrogen receptor α at the plasma membrane. Mol. Cell. Biol. **23:**1633–1646.
31. **Razandi, M., P. Oh, A. Pedram, J. Schnitzer, and E. R. Levin.** 2002. Estrogen receptors associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol. Endocrinol. **16:**100–115.
32. **Razandi, M., A. Pedram, S. Parks, and E. R. Levin.** 2003. Proximal events in ER signaling from the plasma membrane. J. Biol. Chem. **278:**2701–2712.
33. **Rebbeck, T. R., A. M. Levin, A. Eisen, C. Snyder, P. Watson, L. Cannon-Albright, C. Isaacs, O. Olopade, J. E. Garber, A. K. Godwin, M. B. Dal, S. A. Narod, S. L. Neuhausen, H. T. Lynch, and B. L. Weber.** 1999. Breast cancer risk after bilateral prophylactic oophorectomy in BRCA1 mutation carriers. J. Natl. Cancer Inst. **91:**1475–1479.
34. **Robbins, D. J., E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb.** 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J. Biol. Chem. **268:**5097–5106.
35. **Santen, R. J., and G. R. Petroni.** 1999. Relative versus attributable risk of breast cancer from estrogen replacement. J. Clin. Endocrinol. Metabol. **84:**1875–1881.
36. **Santen, R. J., R. X. Song, R. McPherson, R. Kumar, L. Adam, M. H. Jeng, and W. Yue.** 2002. The role of mitogen-activated protein (MAP) kinase in breast cancer. J. Steroid Biochem. Mol. Biol. **80:**239–256.
37. **Scully, R., and D. M. Livingston.** 2000. In search of the tumour-suppressor functions of BRCA1 and BRCA2. Nature **408:**429–432.
38. **Shao, N., Y. L. Chai, E. Shyam, P. Reddy, and V. N. Rao.** 1996. Induction of apoptosis by the tumor suppressor protein BRCA1. Oncogene **13:**1–7.
39. **Singer, C. A., X. A. Figueroa-Masot, R. H. Batchelor, and D. M. Dorsa.** 1999. The mitogen activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J. Neurosci. **19:**2455–2463.
40. **Somasundaram, K., H. Zhang, Y.-X. Zeng, Y. Houvras, Y. Peng, H. Zhang, G. S. Wu, J. D. Licht, B. L. Weber, and W. S. El-Deiry.** 1997. Arrest of the cell cycle by the tumor-suppressor BRCA1 requires the CDK-inhibitor p21waf1/CiP1. Nature **280:**187–189.
41. **Stoica, G. E., T. F. Franke, A. Wellstein, F. Czubayko, H. J. List, R. Reiter, E. Morgan, M. B. Martin, and A. Stoica.** 2003. Estradiol rapidly activates Akt via the ErbB2 signaling pathway. Mol. Endocrinol. **17:**818–830.
42. **Stratton, M. R., D. Ford, S. Neuhasen, S. Seal, R. Wooster, L. S. Friedman, M. C. King, V. Egilsson, P. Devilee, R. McManus, et al.** 1994. Familial male breast cancer is not linked to the BRCA1 locus on chromosome 17q. Nat. Genet. **7:**103–107.
43. **Stuart, N. S., J. Warwick, G. R. Blackledge, D. Spooner, C. Keen, A. R. Taylor, C. Tyrell, D. J. Webster, and H. Earl.** 1996. A randomized phase III cross-over study of tamoxifen versus megestrol acetate in advanced and recurrent breast cancer. Eur. J. Cancer **32:**1888–1892.
44. **Svegliati-Baroni, G., F. Ridolfi, A. Di Sario, A. Casini, L. Marucci, G. Gaggiotti, P. Orlandoni, G. Macarri, L. Perego, A. Benedetti, and F. Folli.** 1999. Insulin and insulin-like growth factor-1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. Hepatology **29:**1743–1751.
45. **Tomlinson, G. E., T. T.-L. Chen, V. A. Stastny, A. K. Kirmani, M. A. Spillman, V. Tonk, J. L. Blum, N. R. Schneider, L. I. Wistuba, J. W. Shay, J. D. Minna, and A. F. Gazdar.** 1998. Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. Cancer Res. **58:** 3237–3242.
46. **Venkitaraman, A. R.** 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell **108:**171–182.
47. **Watters, J. J., T. Y. Chun, Y. N. Kim, P. J. Bertics, and J. Gorski.** 2000. Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. Mol. Endocrinol. **14:**1872–1881.
48. **White, R., and M. G. Parker.** 1998. Molecular mechanisms of steroid hormone action. Endocr.-Rel. Cancer **5:**1–14.
49. **Xu, X., Z. Weaver, S. P. Linke, C. Li, J. Gotay, X.-W. Wang, C. C. Harris, T. Ried, and C.-X. Deng.** 1999. Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol. Cell **3:**389–395.
50. **Yan, Y., J. P. Haas, M. Kim, M. K. Sgagias, and K. H. Cowan.** 2002. BRCA1-induced apoptosis involves inactivation of ERK1/2 activities. J. Biol. Chem. **277:**33422–33430.
51. **Yu, Q., Y. Geng, and P. Sicinski.** 2001. Specific protection against breast cancers by cyclin D1 ablation. Nature **411:**1017–1021.
52. **Zhang, X., and D. Yee.** 2000. Tyrosine kinase signaling in breast cancer: insulin-like growth factors and their receptors in breast cancer. Breast Cancer Res. **2:**170–175.
53. **Zhang, Z., B. Maier, R. J. Santen, and R. X. Song.** 2002. Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. Biochem. Biophys. Res. Commun. **294:**926–933.
54. **Zheng, L., L. A. Annab, C. A. Afshari, W.-H. Lee, and T. G. Boyer.** 2001. BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. Proc. Natl. Acad. Sci. USA **98:**9587–9592.